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Identification and characterisation of differentially displayed transcripts in metastatic versus non-metastatic cells of the CSML cell line system

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**Identification and characterisation of differentially
displayed transcripts in metastatic versus non-
metastatic cells of the CSML cell line system.**

A thesis submitted fulfilling the requirements of the

Open University for the degree of

Doctor of Philosophy

Fabritius

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Abstract:

Metastasis is a complex multistep process allowing otherwise localised tumour cells to migrate to other organs. To elucidate this mechanism further we compared the mRNA level of non-metastatic CSML-0 to metastatic CSML-100 and VMR-Li using differential display. This approach led to the identification of ten different genes differentially expressed between two closely related CSML cell lines. Cloning, sequencing and database analysis suggested the cloned fragments to be related to six genes with known coding sequence. MMTV, Collagen XI α 1 and PACE4 were found in CSML-0, while Cystatin C, Lamin C and Ly-6A/E were upregulated in CSML-100. Also four fragments, NN18 found in CSML-0 and NN9, NN30 and NN32 found in CSML-100, not related to any known gene were cloned. When tested against a panel of mouse tumour cell lines, a pattern of expression for Collagen XI α 1, PACE4, Cystatin C, Lamin C, Ly-6A/E, NN9 and NN30 connecting these genes to the metastatic process were found. Cystatin C protein staining of cell lines and CSML tumour tissue further suggested an involvement of this proteinase inhibitor in tumour progression. This is the first report connecting PACE4 and Collagen XI α 1 to tumour progression and points to new proteins potentially utilised by colonising cells. The NN30 gene fragment was selected for further identification and characterisation. The full-length clone revealed a 338 amino acid 39 kDa protein, lacking obvious homologues in the EMBL database. We temporarily named this protein E30. *In situ* hybridisation and immunostainings demonstrated that this protein was highly expressed in epithelial cells, in testis and in ovary, in embryonic tissue as well as in extra embryonic. This gene is part of a previously undescribed gene family as indicated by human and *C. elegans* ESTs homologous to E30. Transfection of E30 into non-metastatic CSML-0 cells rendered these cells metastatic in 2 out of 3 mice in an experimental metastasis assay. Metastatic nodules were not only confined to the lung, but both mice produced liver metastasis. I therefore report the cloning of a gene expressed in epithelial cells and capable of inducing metastasis to the liver and lung.

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Abbreviations:

aa	amino acids
APC	adenomatous polyposis coli
DAP	death-associated protein
DD	differential display
dpc	days post coitum
ECM	extracellular matrix
EGFR	epithelial growth factor receptor
EMBL	European molecular biology laboratory
EST	expressed sequence tag
FAK	focal adhesion kinase
FAP	familial adenomatous polyposis
GST	Gluthathione-S-transferase
HA	hyaluronic acid
HE-stain	hematoxylin-eosin stain
HGF	hepatocyte growth factor
ICAM	intercellular adhesion molecule
iv	intravenous
kDa	kiloDalton
MAP	mitogen-activated protein
MMP	matrix metalloproteinase
MMTV	mouse mammary tumour virus
orf	open reading frame
PA	plasminogen activator
PACE	paired amino acid cleaving enzyme
PAI	PA inhibitor
PCR	polymerase chain reaction
sc	sub cutis
TGF	transforming growth factor
TIMP	tissue inhibitors of metalloproteinases
tPA	tissue type PA
uPA	urokinase PA
uPAR	uPA receptor
VCAM	vascular cellular adhesion molecule

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1. Introduction

Mechanism of metastasis is poorly understood

Metastasis is a multistage process, requiring a number of abilities of the colonising cell, abilities not possessed by any one normal cell. The metastatic cell must 1) dissociate from the primary tumour, 2) make its way through the extracellular matrix (ECM), 3) intravasate to either the blood stream or lymphatic system, 4) survive unattached, while avoiding the immune defence, 5) stop at or adhere to a proper site, extravasate into foreign tissue and 6) proliferate, generate blood supply etc. therein (1). Though, the events leading to the appearance of the cancer cell are being increasingly understood the behaviour of the metastatic cell is comprehended to a much lesser extent. The reasons for this are many. Firstly, when looking at tumour cells and their ability to proliferate the interplay between the tumour cell and the surrounding tissue is less complex, simplifying the system being investigated. Also, several pathways used by the detached cells are available making conclusions dependent on the system investigated. It is likely that the pathways used by the metastasising cell depend on the origin of the tumour, i.e. the location in the organism and the origin of the cell. However, even when this is taken into account general conclusions have been difficult to make.

Several natural processes mimic the mobile and invasive behaviour acquired by the metastatic cell. These include acquisition of abilities necessary for keratinocytes during wound healing, mesenchymal cells during embryogenesis, trophoblast to form the placenta and lymphocyte migration. The transitions following induction of the abilities of these cells lead to changes for example from an epithelial-like to a more anaplastic mesenchymal like morphology. Some sort of master switch or switches inducing these transformations in normal cells could be activated during malignant transformation, thus enabling the malignant cell to move to and invade foreign tissue. However, this remains to be proven.

It is difficult if not impossible to separate the functional processes necessary for tumourigenesis and metastasis. Anyway, in order to describe the requirements for the metastatic process, proteins crucial for this process have been identified. The

identified proteins have roughly been divided into five groups: 1) Proteins working in adhesion primarily thought to sequester the tumour cells in the tumour, but e.g. in the case of integrins also believed to be essential for the homing of the malignant cell. 2) Proteolytic enzymes shown to be in action during intravasation and perhaps during extravasation breaking down the basal membrane enabling cells to escape into and out of the blood stream. 3) Proteins involved in the mobility of cells, since it is generally believed that in order to spread from one site to another, the cell must have the ability to move by its own force. 4) Once in the bloodstream cells are prone to anoikis, the process of apoptosis liable for unattached cells. It is therefore likely that not only the cells inside the primary tumour, but also the metastatic cell must be protected against apoptosis. 5) Proliferation at the secondary site probably requires either the ability to utilise growth factors residing at this site or independence from otherwise vital factors in the environment.

This is not a comprehensive overview of all the knowledge concerning metastasis, since the amount of literature on the subject is much too overwhelming. Rather it is a subjective choice of biological proteins or protein systems thought to play a role in metastasis. The object of the thesis was to identify differences between two cell lines with different metastatic potential. This angle of approach leads to focus on single proteins functionally involved in this process.

Adhesion

Almost 90% of all tumours are of epithelial origin, a group of cells containing adhesion molecules binding with strong homotypic affinity. During the outgrowth of the primary tumour these adhesion proteins are thought to keep the cells together. However, cell adhesion serves opposite functions during tumour progression. On one site adhesion is thought to maintain non-metastasising cells inside the primary tumour thereby avoiding metastasis, on the other some adhesion molecules are possibly required for motility, homing and growth stimulation. Though many more adhesion molecules have been described only E-cadherin, the integrins and CD44 will be described here. The best-investigated example of a cellular adhesion molecule negatively controlling the progress of the tumour is E-cadherin.

E-cadherin

E-cadherin is part of the cadherin family of calcium dependent adhesion molecules consisting of more than 30 members. The 135kDa precursor is processed to yield the 120kDa mature protein. Extracellularly five CAD repeats are encountered containing two conserved regions, which are the putative calcium-binding sites (2; 3). The outermost domain contains an HAV-sequence essential for homophilic recognition also found in other cadherins, although flanked by other amino acids. The 31 amino acids comprising the transmembrane region connect the extracellular part to the highly conserved cytoplasmic domain (4). A 30 amino acid region comprises the recognition site for catenins intracellularly (4; 5). The tail complexes two sets of catenin: one complex consists of α -catenin, γ -catenin and E-cadherin, while the other is composed of α -catenin, β -catenin and E-cadherin (6; 7). Another catenin-like protein, p120-cas, has been found to be part of these complexes (8). Catenins are believed to form the link between the actin cytoskeleton and E-cadherin. First, it seems, β -catenin binds E-cadherin, while it is being transported to the plasma membrane, thereafter α -catenin connects this complex to actin (7; 9; 10). This complex is in the normal cell part of the adherens junctions and expressed at the basolateral surface of epithelial cells (11).

Two types of studies have implicated E-cadherin in metastasis. Firstly, expression studies of invasive tumours indicate a downregulation. Secondly, experimental up- and downregulation of E-cadherin lead to inhibition and induction of invasion, respectively. Downregulation of E-cadherin has been found in a number of carcinomas. As a general rule E-cadherin downregulation was correlated with malignancy parameters, such as tumour progression, loss of differentiation, invasion, metastatic potential and poor prognosis (12). Also, transfection experiments inducing expression of E-cadherin into otherwise non-expressing cell lines inhibited invasion and metastasis (13; 14), while antisense expression downregulating E-cadherin expression induced invasion (14). Induction of invasion could also be achieved by the use of monoclonal antibodies against E-cadherin (13; 15). Though the expression of E-cadherin therefore closely correlates with metastasis, metastatic E-cadherin cell lines have been reported, indicating its functional inactivation. This could be caused by mutations impeding adhesion or function of the cytoplasmic tail, or could be

caused by downstream regulators, like the catenins. In vitro it was shown that mutations in domains involved in complex formation with the catenins (9) in calcium binding (2) or in enzymatic processing of the precursor protein (16) impaired aggregation and/or compaction.

Though point mutations are infrequent, mutations in exon 7, 8, 10, 12 and 13 representing the extracellular part of E-cadherin and in exon 16, a part of the cytoplasmic tail have been described for some carcinomas (12). As mentioned this could lead to a non-functional E-cadherin. Also downregulation of one of the catenins could lead to E-cadherin inactivity as described for α -catenin (17) and β -catenin (10; 18; 19).

E-cadherin functions via catenins, a complex of E-cadherin, α -catenin and β -catenin or γ -catenin (plakoglobin) binds the actin cytoskeleton. β -catenin was found to be a close homologue of armadillo a drosophila gene part of the wingless pathway (20) and seems to be a component of the homologous mammalian wnt pathway active during tumour progression (21). β -catenin was also shown to associate with the protein adenomatous polyposis coli (APC), thereby regulating the intracellular β -catenin level (22; 23). APC has been found to be truncated in the hereditary colon cancer familial adenomatous polyposis (FAP) and β -catenin does not bind truncated APC (22). A regulatory function of β -catenin was indicated when it was found that it binds the transcription factor tcf/lef-1, leading to translocation of β -catenin to the nucleus (24-26).

All in all there is compelling evidence for the involvement of functional E-cadherin in prevention of cancer metastasis. However, the mere homophilic adhesion of E-cadherin is not sufficient, as intracellular proteins mediate binding to the actin filaments and participate in signalling. At least β -catenin seems to be an important moderator of E-cadherin adhesion.

Integrins

While E-cadherin is believed to impede spread of cancer cells, other adhesion proteins, like the integrins have more complex functions. These adhesion proteins are a growing family of heterodimeric integral plasma membrane proteins mediating adhesion to the ECM or other cell surface proteins most of which belong to the immunoglobulin superfamily (27-29). Each heterodimer is composed of one α and one β subunit. Though, more than 16 α and 7 β units have been discovered, theoretically allowing more than 100 combinations, only a little more than 20 of these

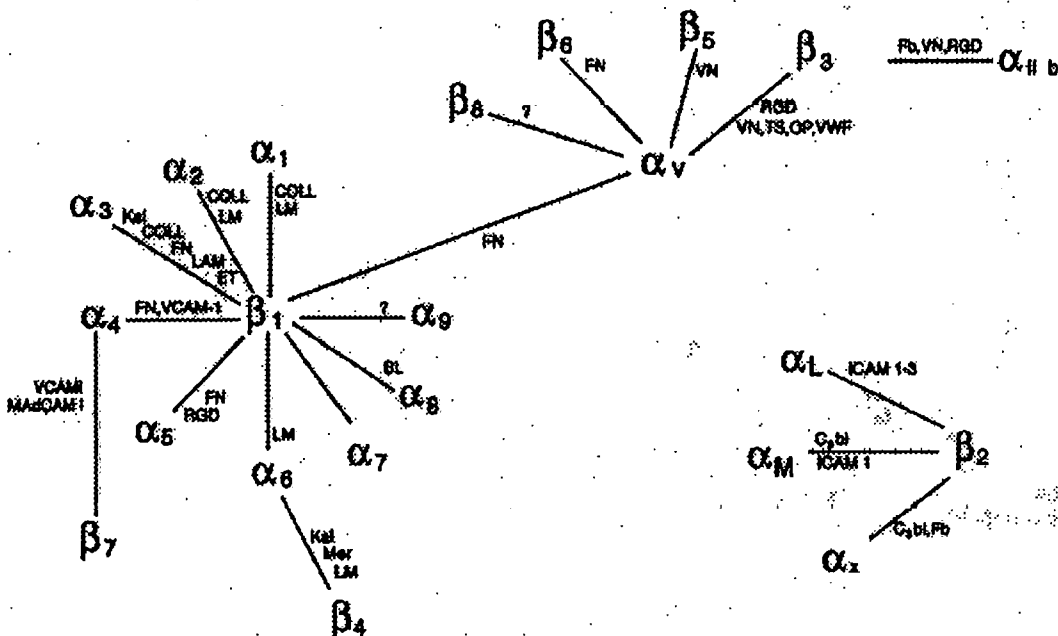


Figure 1.1: The integrin receptor family. A schematic representation of various $\alpha\beta$ integrin heterodimers and the ligands they interact with. COLL: collagen; FN: fibronectin, LM: laminin, ET: entactin, Kal: kalikvein, Mer: merosin, BL: basal lamina, VCAM: vascular cell adhesion molecule, ICAM: intercellular cell adhesion molecule, F6: fibrinogen, VN: vitronectin, TS: thrombospondin, OP: osteopontin, VWF: Von Willebrand factor, RGD: ArgGlyAsp-peptide, MadCAM mucosal address in cell adhesion molecule. From (31)

combinations have been described (Fig. 1.1). The α and β subunits contain an extracellular domain much larger than the carboxy-terminal intracytoplasmic domain except for $\beta 4$, due to the very large cytoplasmic domain of this subunit (30). Each of the identified integrins has a unique recognition pattern, binding either ECM components like fibronectin, collagen or laminin, or mediating adherence to other cells via e.g. VCAM-1 or ICAM (31).

The sequence recognised most frequently consists of the integrin-binding site RGD, but depends on the specific combination of α and β subunits. For example all α -v

containing integrins have been shown to bind this site, while no β -2 containing integrin does (32).

While integrins in general are ubiquitously expressed, there are variations in the expression of the specific integrin. The most promiscuous partner, integrin β -1, that mediates binding to the ECM, is ubiquitously expressed, while the integrin α -4 β -1 is unique among the β -1 integrins in that it is restricted in its expression to bone marrow-derived cells, except for neutrophils (33) on certain tumour cells and in muscle development (34). The leukocyte-specific integrin α -E β -7 binds E-cadherin and this may represent a mechanism by which these lymphocytes remain in their respective epithelial tissue (35).

Binding not only mediate adhesion. Rather integrin-binding set off a number of intercellular events leading to survival, motility and proliferation of normal and tumour cells. Central to the many roles that integrins play is integrin-mediated signal transduction processes. Evidence is surfacing that integrins can activate the MAP-kinase pathway, through which several nuclear transcription factors are activated. When fibroblasts attach to fibronectin, MAP kinase is activated (36; 37). This activation could occur via Ras, since activation of integrin α -2 β -1 results in accumulation of GTP-bound Ras (38), and via tyrosine phosphorylated focal adhesion kinase (FAK) (37). Also the stimulation of tyrosine phosphorylation, by insulin, of the insulin receptor substrate, IRS-1, results in the association of phosphorylated IRS-1 to α -v β -3. It was demonstrated that insulin stimulated proliferation led to enhanced proliferation when cells were plated on vitronectin, a ligand for integrin α -v β -3 (39).

Normal epithelial, endothelial cells as well as some tumour cells die when deprived of the ECM. However, constitutive activation of intracellular signal proteins (that are often oncogenes) allows the cells to survive, despite the lack of integrin attachment. Also, overexpression of integrin α -v β -3 on human melanoma cells increased their survival in Matrigel (40). Their increased survival could be due to induced expression of the apoptosis suppressor Bcl-2 (41).

A significant role for integrin α -v β -3 in the process of angiogenesis has been reported. During angiogenesis this integrin was upregulated (42), while antibodies to this protein inhibit angiogenesis, but had no effect on pre-existing vessels.

There is overwhelming evidence that integrins contribute to tumour progression. Integrin α -v β -3 are required for metastasis of melanoma cells (43-46). Expression of this promiscuous integrin allows the cell to respond to almost any matrix protein it may encounter. Also, integrin α -v β -3 has been shown to bind MMP-2 in an active form thereby localising this protease on the surface of invasive tumour cells (47). The integrin α -2 β -1 have been associated with metastasis of human rhabdomyosarcoma cells inducing both experimental and spontaneous metastasis (48). Association of MMP-1 expression to integrin α -1 β -2 expression has also been suggested (49). The involvement of β -1 integrin during metastasis was confirmed, since the disruption of integrin α -4 β -1 and integrin α -6 β -1 function reduces the metastatic capability of highly metastatic ESb murine T-lymphoma cells (50). Curiously, this changed the organ preference of the metastasising cells from spleen and liver in highly metastatic non-mutated ESb cells to skeletal muscle in reduced metastatic ESb knock out mutants.

CD44

CD44 is another transmembrane adhesion protein implicated in metastasis (51). The extracellular part has been shown to bind a variety of proteins but is generally accepted as a hyaluronic acid (HA) acceptor (52). HA is a polysaccharide consisting of a linear polymer of repeating disaccharide units of high molecular weight (10^6 - 10^7 kDa) and an integral part of the ECM. CD44 is a glycoprotein built of 10 exons in its standard form (CD44s), where the primary 7 exons are extracellular and exon 8 contains the transmembrane region. One of the last two exons code for the cytoplasmic tail: exon 9 represents the short tail form of CD44 containing a cytoplasmic tail of only 3 amino acids, while transcripts containing exon 10 results in a protein with a cytoplasmic tail of 70 amino acid. These 70 amino acids have been shown to contain a number of phosphorylation sites of which at least two are constitutively phosphorylated. The tail of CD44 has been shown to interact with the microfilament through ezrin-radixin-moesin (53). A number of splice variants has

been described in which 10 different inserts (CD44V1-10) inserted after exon 5 have been identified e.g. the epithelial form containing V8-10 and the keratinocytic form containing V3-10.

CD44 was initially discovered as a brain-granulocyte-T lymphocyte antigen and connected to metastasis, when CD44 high expressing cells sorted from low-expressing cells revealed a higher metastatic capability (54). The most convincing evidence comes from transfection of non-metastatic rat pancreatic tumour cells with the CD44 variant form pMeta-1 (CD44V4-7) or pMeta-2 (CD44V6-7) (55; 56). This induces the ability to form lung nodules 40-60 days after injection sc of 5×10^5 cells.

Proteolysis

An early observation of tumour tissue connected to tumour progression was the degradation of the basement membrane. This has been shown to involve one or more proteases controlled by their corresponding inhibitors. Several proteolytic systems thought to be involved in invasion and metastasis have been described, involving proteases of all five major classes (i.e. the serine, aspartic, cysteine, threonine, and metalloproteinases). The major enzymes in each of these classes of proteases seem to be the serine protease urokinase plasminogen activator (uPA), the matrix metalloproteinases MMP-2 and MMP-9 and the cysteine proteinase Cathepsin B.

Plasminogen activation system

During the past decade increasing evidence has evolved, implicating uPA in cancer metastasis. There appears to be general agreement on the notion that the related tissue type plasminogen activator (tPA) is primarily involved in thrombolysis. The reaction pathway of uPA involves its uPA receptor (uPAR), the plasminogen activator inhibitors (PAI-1 and PAI-2), controlling the activation of plasminogen to plasmin. The major target of plasmin is fibrin, but several studies have shown a broad spectrum of degradation of the ECM components like fibronectin and vitronectin (57). Also plasmin has been shown to activate other matrix degrading enzymes e.g. metalloproteinases. Activation of uPA has been shown to be enhanced when bound to uPAR. This activated pair can be inhibited by PAI-1, that when bound promotes endocytosis of this complex. Staining of tumour tissue for members of this system

shows PAI-1 expressed inside the tumour, while uPA and uPAR very often are situated at the invasive front (58; 59). The uPAR or uPA can be expressed by the cancer cells or the surrounding stroma cells, thereby assisting in breakdown of the ECM and tumour invasion.

Transfection experiments introducing uPAR into rat breast cancer cells (60) or antisense inhibiting uPAR expression in human epidermoid carcinoma cell line (61), respectively elevated and reduced metastatic capacity of the transfected cells.

Elevating the expression of uPAR increases the number of metastatic nodules with a factor 3 to 5 and allows for metastasis formation in liver and spleen, organs otherwise not invaded. uPA expression also influences the metastatic capacity of murine B16 melanoma cells (62) and rat prostate cancer cells (63). Increased metastatic formation in the lung and skeletal muscle correlated with elevated levels of uPA in these cells. Furthermore, downregulation of uPA in metastatic B16-F10 cells reduced number of lung colonies formed. These notions support the involvement of uPA and uPAR in metastasis. However, other proteolytic systems connected to the uPA system play important part in tumour progression as well. One of these systems are the gelatinases MMP-2 and MMP-9.

Matrix metalloproteinases (MMPs)

The MMPs are a family of zinc dependent, secreted or transmembrane proteases able to degrade components of the ECM and the basement membrane (Fig. 1.2) originally identified as enzymes responsible for the dissolution of the tadpole tale. The proteases are exported as zymogens awaiting further extracellular activation. They are divided into three major subgroups by their substrate specificities: collagenases (MMPx-y) degrade fibrillar collagen, stromelysins (MMPz-u) prefer proteoglycans and glycoproteins, while the gelatinases (MMP-2 and MMP-9) degrade nonfibrillar and denatured collagen (gelatine) (64). Once activated the MMPs are susceptible to inactivation by a number of inhibitors, most notably one of the four known tissue inhibitors of metalloproteinases (TIMPs).

The MMPs has been associated to the metastatic process for more than 15 years (65; 66) and further sustained to date (67). These investigations demonstrates that the number of MMP family members found tends to increase with progression and that

the MMPs detected could be expressed by the tumour cells, but also by the

MMP	DOMAIN STRUCTURE	MAJOR SUBSTRATES
Matrilysin (MMP-7, pump-1) (EC 3.4.24.23)	PRE PRO	Proteoglycans, ECM glycoproteins, IV collagen, gelatins, elastin
Interstitial Collagenase (MMP-1, EC 3.4.24.7)	PRE PRO	Fibrillar Collagen
Neutrophil Collagenase (MMP-8, EC 3.4.24.34)	PRE PRO H HEM	Fibrillar Collagen
Collagenase-3 (MMP-13)		Fibrillar Collagen
Stromelysin-1 (MMP-3, transin, EC 3.4.24.17)		Proteoglycans, ECM glycoproteins, IV collagen, gelatins
Stromelysin-2 (MMP-10, transin-2, EC 3.4.24.22)		Proteoglycans, ECM glycoproteins, IV collagen, gelatins
Metalloelastase (MMP-12, EC 3.4.24.65)		Elastin
MMP-18		N.D.
MMP-19		N.D.
Stromelysin-3 (MMP-11)	PRE PRO F H HEM	Laminin and fibronectin (weakly)
MT1-MMP (MMP-14)		Gelatinase A, fibrillar collagens, proteoglycans, ECM glycoproteins
MT2-MMP (MMP-15)	PRE PRO F H HEM TM	N.D.
MT3-MMP (MMP-16)		Gelatinase A
MT4-MMP (MMP-17)		N.D.
Gelatinase A (MMP-2, 72kD gelatinase, IV collagenase, EC 3.4.24.24)	PRE PRO FN H HEM	Gelatins, collagen IV, collagen I
Gelatinase B (MMP-9, 92kDa gelatinase, IV collagenase, EC 3.4.24.35)	PRE PRO FN CH HEM	Gelatins, collagen IV, collagen V

Figure 1.2: The family of matrix metalloproteinases. Subgroups are arranged by domain structure and separated by a dashed line. Within the largest, hemopexin-domain subgroup, family members with some distinct but subtle structural features are separated by a dotted line. MT: membrane type, PRE: leader sequence, PRO: prodomain, H: hinge domain, HEM: hemopexin-like domain, F: furin consensus site, FN: fibronectin-like domain, TM: transmembrane domain, N.D.: not determined. From (64).

surrounding stromal cells.

From early on the focus was on proteinases degrading type IV collagen, a major structural protein in the basement membrane, that is the MMP-2/gelatinase A/72 kDa type IV collagenase and MMP-9/gelatinase B/92 kDa type IV collagenase. The primary purpose of these proteases in tumour malignancy (along with other proteolytic enzymes) is thought to be the breakdown of the physical barrier between the primary tumour and sites of metastasis, i.e. dissolution of the basement membrane during intravasation and extravasation. MMP activity has been shown to be linked to the invasive potential of tumours through detection in metastatic cell lines (68; 69).

Moreover the elevated collagenolytic activity correlates with stage in colon and bladder cancer (70; 71).

Activation of proMMP-2 through MT1-MMP surface binding and integrin α -v β -3 (47; 72) localises this system to the membrane indicating an involvement in metastasis formation. Indeed, overexpression of MMP-2 and MMP-9 in non- or low-metastatic cell lines induces the metastatic phenotype (73-76). Inhibition of tumour invasion could be obtained using Marimastat (BB-2516) a protease inhibitor blocking proteolysis (77). However, natural inhibitors regulate the activity of the MMPs and changing the quantity of inhibitors also affect metastasis formation.

Manipulation of the TIMP-1 and TIMP-2 expression level using antisense or sense transfections indicates a role for these inhibitors in tumour progression. Reducing the level of TIMP-1 in Swiss 3T3-cells induced the formation of metastatic tumours in nude mice (78), while overexpression reduced metastatic ability of human gastric KKLS cancer cells to the chick embryo (79) and of B16F10-cells (80). Also Schultz et al. (81) demonstrated the ip injection of recombinant TIMP-1 reduced formation of lung nodules by B16F10 melanoma cells. However, there are examples of systems in which TIMPs have no effect or enhance tumour growth and/or metastasis (82) and conversely, that TIMP-1 and TIMP-2 also show growth promoting effect (64; 83).

Cathepsin B

Also the cysteine protease Cathepsin B has been implicated in cancer metastasis (84). This ubiquitously expressed proteinase was identified as a lysosomal enzyme secreted under physiological and malignant conditions (85; 86). The protein is often secreted as an inactive protein and inhibited by members of the cysteine protease inhibitor family, most notably Stefin A (Cystatin A), Stefin B (Cystatin B) and Cystatin C. The ability to degrade ECM proteins (87) such as collagen and laminin and activate uPA (88) points to a possible role during invasion and tumour progression. Localisation of the protease to the plasma membrane (89-91) and to the invasive edge of tumours supports this notion (92). Increasing expression of Cathepsin B in murine B16 melanoma cells correlates with metastatic ability of these cells (93; 94). Clinical investigations indicate activity of Cathepsin B to be part of the invasive process,

demonstrating elevated expression of Cathepsin B in e.g. colon carcinoma (95), lung (96) and breast carcinoma (85). As mentioned the inhibition of Cathepsin B could be performed by the intracellularly expressed Stefin A or B or by the secreted Cystatin C. Changing the ratio of the proteolytic activity of Cathepsin B against the activity of the cysteine protease inhibitors shows that murine melanoma B16 cell populations enriched 4-10 times in their Cathepsin B activity also displayed higher metastatic capability (97).

Role of proteases in extravasation

By inference it has been assumed that the proteolytic activity performed by these enzymes was as important for extravasation and intravasation since both processes seems to require break down of the basement membrane. Metastatic B16F10 melanoma cells transfected with TIMP-1 were inhibited in their ability to form metastatic nodules of the lung (80). This has been explained with a possible reduced ability to invade the lung tissue i.e. extravasate into the lung. Similar results and conclusions have been obtained with Cystatin C transfection of B16 cells (98). While additionally considering the inhibition of tumour growth along with the hampered metastatic ability of TIMP-transfected cells, the rate-limiting step in this context could shift from extravasation to tumour growth. However, using intravital videomicroscopy it was demonstrated that B16F10 melanoma cells transfected with TIMP-1 were shown to be growth limited but not restricted in extravasation (99; 100).

Induction of migration

As the tumour grows and progresses in many instances morphology of the cells change from epithelial to more mesenchymal. The hepatocyte growth factor (HGF) has been shown to induce such a change through binding to the MET receptor (101).

HGF

Several independent different groups discovering the ability of HGF to induce growth of hepatocytes and mobility of epithelial cells initially described HGF (also called scatter factor)(102; 103). HGF is secreted as an inactive single chain 100kDa protein, and until now the two proteases uPA and factor XII have been identified as possible *in vivo* activators of HGF (104; 105) cleaving pro-HGF into the active $\alpha\beta$ -HGF. The

active HGF then binds the MET receptor (106) and induces 1) release of the cell, 2) invasion, 3) production of proteases capable of ECM and basement membrane degradation, 4) growth and 5) polarisation in epithelial cells (107). The response to HGF is transient and *in vitro* the affected cells scattered by HGF eventually rejoin.

Several lines of evidence implicate the expression of either HGF or its receptor in metastasis. Transfection experiments with NIH3T3 and C127 rendering them HGF and MET positive allowed for experimental metastasis to the lung (108; 109). It was also shown that the rare “spontaneous” lung metastasis produced in this assay by untransfected NIH3T3 cells overexpressed MET. In human breast cancer it was found that HGF is a predictor of recurrence and survival (110).

Mts1

Motility requires reorganisation of the cytoskeleton involving actin and myosin binding proteins e.g. Mts1 (S100A4). The Ca^{2+} -binding protein was initially isolated from metastatic CSML-100 cells and shown not to be expressed in non-metastatic and low-metastatic cell lines (111). This gene product is being implicated in motility and invasion, since it is expressed in murine and human macrophages, T-lymphocytes, neutrophils and monocytes, but not in B-cells, which are non-invasive. S100A4 proteins of different species has been shown to associate to cytoskeletal proteins i.e. non-muscle myosin, non-muscle tropomyosin or actin (112-114). This possible involvement in motility has encouraged investigations into metastatic potential of Mts1. Transfection of various cell systems induce metastasis in otherwise low-metastatic cell lines (115-117), while antisense expression reducing the level of Mts1 protein impedes metastasis of otherwise low-metastatic cells (118) correlating with suppression of motility (119). The most compelling evidence for the involvement of Mts1 in metastasis comes from experiments with transgenic mice (120; 121). No alterations in phenotype were seen in transgenic mice expressing elevated amounts of Mts1 in the mammary gland. However, crossing these mice with GRS/A mice, who have high incidences of benign mammary tumours, lead to lung metastasis in 40% of the tumour bearing hybrids.

Apoptosis in metastasis

Though described in 1972 (122), the importance of programmed cell death was not recognised until the beginning of the '90. Since then, the field has developed explosively e.g. involving embryogenesis, tissue remodelling, tumour development and recently data has evolved implicating resistance to apoptosis in tumour progression.

Resistance to apoptosis promotes metastasis

One of the rate limiting steps in metastasis is thought to be survival of the unattached cell following intravasation. It is straightforward, that survival in the blood stream would require the ability to resist attack from the immune system and apoptosis resulting from the detachment from tissue (ECM, basal lamina, collagens). However, only few examples are described until now. DAP kinase was isolated using a functional cloning technique isolating genes involved in interferon γ induced apoptosis (123). DAP kinase is a 160kDa calmodulin dependent serine/threonine kinase, located at the actin filamentous part of the cytoskeleton, containing a C-terminal death domain (124). Its expression is often lost in human B-cell lymphoma cell lines (125). When comparing murine Lewis (3LL) and CMT64 lung carcinoma cell lines for DAP kinase expression low metastatic cell lines expressed higher amounts than their highly metastatic counterparts. Transfection experiments revealed a reverse correlation between level of expression and metastatic capability in 3LL cells, while sensitivity to TNF α induced apoptosis increased (126). Analogous results have been reported by Shtivelman (127), who, using differential display (DD), isolated CC3 from low metastatic human c-SCLC cell lines. Highly metastatic v-SCLC cell lines did not express this gene. The ability to metastasise to grafts of human foetal lung in SCID-hu-L mice was almost completely abolished for CC3 transfected v-SCLC cells. This was explained by a higher susceptibility to apoptosis since TUNEL staining of tumours and cultivation transfected cells in 0.5% serum increased apoptosis. Using transgenic mice expressing Bcl-x_L under the control of the keratin 14 promoter it was shown that the affected mice are more susceptible to chemical induction of tumours and that these tumours frequently undergo malignant conversion (128). These reports are in agreement with the generally believed conception of metastasis, where the stress imposed on the evading cell after

detachment from the ECM and in circulation must require greater resistance to apoptosis. The contrary was demonstrated, when transfecting EGFR into low metastatic rat mammary adenocarcinoma MTC cells (129). This made the transfected cells more susceptible to apoptosis, while increasing their metastatic potential. Apoptosis was linked to cell cycle stage, since TGF α treatment halved the amount of cells in G₂/M. Hence, expression of proteins protecting metastatic cells from apoptosis, while in the process of spreading (Bcl-x_L and EGFR) are upregulated during metastasis. Expression of proteins typical of low-metastatic cells impeding metastasis (DAP kinase and CC3) on the other hand is reduced.

Induction of apoptosis promotes metastasis

While the metastatic cells are thought to resist apoptosis, metastatic colonic adenocarcinomas have been suggested to induce apoptosis by production of the Fas ligand. The Fas ligand is expressed in immunologically privileged sites in the body and thought to play a role in deletion of activated T lymphocytes at these sites (130). The ligand was also present on the metastatic cells invading the liver and suggested to induce apoptosis in T lymphocytes and in Fas expressing hepatocytes.

Growth induction in target organs

Survival of the detached cells enables it to circulate to a distant site and adhere specifically to the endothelial cells of the organ or get trapped in the capillaries of e.g. the lung. This arrest could lead to invasion into the organ and proliferation. However, many tumour cells are not capable of proliferation in the foreign tissue. There is an incompatibility between the seed and the soil.

The explanation for this could be several, one being that the foreign tissue lacks growth factors crucial to the invading cells. Cultivation of prostate carcinoma cells with conditioned media from fibroblasts of different tissue pointed to bone fibroblasts as growth stimulatory. Stimulation of the cells was mutual since conditioned media from the carcinoma cells stimulated the bone fibroblasts. This is of interest since bone is a common metastatic site of prostate cancer (131). In the same line of evidence highly metastatic 3LL Lewis Lung carcinomas express high amounts of the PDGF α -receptor, while low metastatic clones downregulated this receptor. Up or

downregulation of this receptor in low or high metastatic clones, respectively, enhanced or reduced the metastatic capability in the affected cells (132). Several other lines of evidence point to specific factors in the organ as determinants for metastasis formation (133; 134). As the primary cells seek to metastasise there seem to be one or several organs of preference. One of the explanations for this preference is probably the ability to grow in an environment of specific growth factors. Further progression of the metastatic cell lines, however, probably leads to a loss of this preference allowing for metastasis to other organs (135).

Objective

As described in all of the above plenty of pathways can lay down the tracks for the cancer cell and lead it to the lung. Many different molecules facilitating adhesion, proteolytic degradation, growth induction etc. can render the affected cell metastatic. Various factors affect the metastatic abilities of each cell, factors that are likely to depend at least on the origin of the cell. This work is an attempt to investigate differences amongst three closely related adenocarcinoma cell lines, non-metastatic CSML-0 and metastatic CSML-100 and VMR-Li, using differential display. I report the identification of mRNA from six known and four unknown genes differentially expressed in a panel of cells with different metastatic potential. The expression of one of these proteins (Cystatin C) was also demonstrated in tumour tissue from CSML-100 as compared to CSML-0. Another of the gene fragments, having no homology to any known gene, was used for cDNA-library screening. The full length clone, temporarily called E30, and characterised using polyclonal antibodies and transfection experiments. While the antibodies suggested that E30 is a protein expressed mainly in the cytoplasm of epithelial tissue, the transfection experiments implicate an involvement of this gene in metastasis.

2. Materials and Methods:

Cell lines: The cell lines used for DD were derived from spontaneous mouse mammary adenocarcinoma, and characterised as a non-metastatic CSML-0, a metastatic CSML-100 (136) and a metastatic VMR-Li cell lines (137). CSML-0 and CSML-100 originated from the same tumour. The panel of cells consisted of five related adenocarcinoma cell line systems, the previously mentioned VMR and CSML, plus MT1 (138), RAC (139) and Miller cells (140) and four additional cell lines, the lung carcinomas Line1 (141) and LL-Met (142) and the fibroblast cell lines NIH3T3 and 10T½ (143). The panel contained three non-tumourigenic cell lines (NIH3T3, 10T½ and RAC311C), five non-metastatic cell lines (VMR-0 p9, CSML-0, RAC10P, 67NR and 168FARN), one cell line metastatic in a spontaneous assay only (MT1TC3), four cell lines displaying metastatic behaviour in the experimental assay only (VMR-0 p25, MT1TC1, RAC34E and RAC5E) and five metastatic cell lines metastatic in both assays (VMR-Ly, VMR-Li, CSML-100, 66CL4, 4T07, 4T1, Line1 and LL-Met). The VMR-0 cells became experimentally metastatic when passaged from p9 to p25. The cell lines were grown in Dulbeccos Modified Eagles Media supplemented with 10% foetal calf serum and 4 mM Glutamax (Life Technology).

Differential display (DD): The DD technique is based on a selective reverse transcription and PCR. Firstly 1/12 of the mRNA population is selected using a polyT primer containing two anchoring nucleotides (in this line of experiments the A and G of the downstream primer T₁₁AG). Secondly this downstream primer in conjunction with an arbitrary decamer upstream primer selects the cDNA fragments actually displayed. DD was performed using the RNAmapping™ kit from GenHunter (Brookline, MA, USA) according to the manufacturer's recommendations and as described (144). Briefly, 0.2 µg total RNA was reverse transcribed using superscript reverse transcriptase (Life Technology) and 5'-T₁₁AG-3' as downstream primer, followed by a PCR using the same downstream primer and one of 32 arbitrary 10-mers (DNA-Technology, Aarhus, DK). Sequences were obtained from GenHunter (primers AP1-AP5), M. Strauss (primers 1-26) or designed for this system (primer E)(Table 2.1). Differentially expressed, reproducible bands were excised from the 6% denaturing polyacrylamid gel, extracted in boiling water, PCR amplified and purified using Qiagen kit (Qiagen, Chatworth, CA, USA).

Table 2.1: Upstream primers used for DD:

Primer #	Sequence	Primer #	Sequence	Primer #	Sequence	Primer #	Sequence
1	5'-TACAACGAGG-3'	9	5'-TCGGTCATAG-3'	17	5'-GATCTGACAC-3'	25	5'-GATCATAGCG-3'
2	5'-TGGATTGGTC-3'	10	5'-GGTACTAAGG-3'	18	5'-GATCTCAGAC-3'	26	5'-GATCTAAGGC-3'
3	5'-CTTTCTACCC-3'	11	5'-TACCTAAGCG-3'	19	5'-GATCATAGCC-3'	E	5'-AGCTACTGAC-3'
4	5'-TTTTGGCTCC-3'	12	5'-CTGCTTGATG-3'	20	5'-GATCAATCGC-3'	AP1	5'-AGCCAGCGAA-3'
5	5'-GGAACCAATC-3'	13	5'-GTTTTCGCAG-3'	21	5'-GATCTAACCG-3'	AP2	5'-GACCGCTTGT-3'
6	5'-AAACTCCGTC-3'	14	5'-GATCAAGTCC-3'	22	5'-GATCGCATTG-3'	AP3	5'-AGGTGACCGT-3'
7	5'-TCGATACAGG-3'	15	5'-GATCCAGTAC-3'	23	5'-GATCTGACTG-3'	AP4	5'-GGTACGCCAC-3'
8	5'-TGGTAAAGGG-3'	16	5'-GATCACGTAC-3'	24	5'-GATCATGGTC-3'	AP5	5'-GTTGCGATCC-3'

RNA isolation and Northern blot analysis: RNA was isolated according to Chomczynski and Sacchi (145). In short, harvested cells were lysed in lysis buffer (4 M guanidiniumthiocyanat, 25 mM sodium citrate, 0.5% sodium sarcosyl, and 0.7% β -mercaptoethanol). After separation using an equal volume of phenol and 1/10 volume of chloroform, the RNA was precipitated in isopropanol and reprecipitated over night in 3.3 M LiCl. The precipitated RNA was treated with DNase, phenol extracted, ethanol precipitated and it was stored in 70% ethanol at -80°C. RNA electrophoresis was performed in a 1% agarose gel containing 6.5% formaldehyde and 1 x MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA) according to (146). Transfer of RNA to Hybond N filters was done by capillary blotting in 20 x SSC (3 M NaCl, 0.3 M sodium citrate). After transfer, the filters were baked for 2 h at 80°C in a vacuum oven. DNA-fragments were labelled using either MegaprimeTM (Amersham, Arlington Height, IL, USA) as described by the manufacturer's or PCR-labelled (18 cycles, 30 sec at 94°C, 2 min at 40°C, 30 sec at 72°C) using 25 μ Ci [³²P]dCTP (Amersham, Arlington Height, IL, USA) and the corresponding primers. The labelled fragments were hybridised in hybridisation buffer (50% formamide, 5 x SSC, 10 x Denhardt's solution, 0.5% SDS and 100 μ g/ml single stranded salmon sperm DNA) at 37°C for 16-36 h. After hybridisation the filters were rinsed in 0.5 x SSC, 0.2% SDS once, then washed for 15 min at 37°C. Optional additional washing was done at 45°C and/or 65°C. Total RNA for the filters using 14 cell lines was adjusted using polyU as described (120).

Cloning and sequencing: Fragments displaying differential expression in Northern blot analysis were cloned into the TA pCRII-cloning vector (Invitrogen, San Diego, CA,

USA). The cloned fragments were sequenced by the use of the sequencing kit from Amersham (Arlington Height, IL, USA). The nucleotide sequences were compared with known sequences in the databases through either FASTA or BLAST (Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA) using the services of the Danish Biobase.

Western blotting: Whole cell lysates were prepared by adding 10 μ l 2 x sample buffer (100 mM TRIS (pH=6.8), 4% sodium dodecyl sulphate (SDS), 0.2% Bromophenol Blue, 20% Glycerol, 10% β -mercaptoethanol) to 10^5 pelleted cells. Cells were boiled for 5 min, and the proteins were separated on 15% SDS-polyacrylamid-gel (SDS-PAGE), and transferred to Hybond C filters by semi-dry electro blotting. Evaluation of blotting efficiency and amounts loaded was done by Ponceau S staining according to the manufacturer's instructions. Primary antibody was applied in TBS-T buffer (2mM TRIS pH=7.6, 137mM NaCl, 0.1% Tween 20) supplemented with 10% FCS and 5% dried milk powder for 1 hour at room temperature. Secondary antibodies used were horseradish peroxidase-conjugated swine anti-rabbit or goat anti-rat immunoglobulin (DAKO, Glostrup, Denmark) at a 1:1000 dilution. For detection, the ECL-kit (Amersham) was used according to the manufacturer's instructions.

λ phage-cDNA library: A cDNA library was constructed from CSML-100 mRNA with the ZAP-cDNA synthesis kit and ligated onto Uni-ZAP XR vectors with the ZAP-cDNA Gigapack III Gold Cloning kit (Stratagene). Two plates with 150.000 plaques/plate were blotted onto Hybond N filters (Amersham) and screened using the PCR-labelled DD fragment. cDNA clones were excised as pBluescript phagemids with ExAssist Helper Phage (Stratagene). The cDNA clones were sequenced upstream and downstream using the sequencing kit, v2 (USB).

In situ hybridisation: Virgin A/Sn females were allowed to mate overnight and the morning thereafter denoted as 0.5 dpc. Slides were prepared as described from embryos of stage 12.5, 14.5 and 16.5dpc and from adult mouse organs (147). As specific probe the 1137bp E30 λ phage-cDNA library fragment was excised with EcoRI and cloned into the pRc/CMV vector (Invitrogen). The pRc/CMV-10 vector was linearised with XbaI or HindIII and RNA transcribed as described (147) using the appropriate T7- or

SP6-polymerase to produce the sense and antisense probe. *In vitro* transcription, hybridisation and washing were done as described (147) with an additional step involving alkaline hydrolysis for 52 min. After washing all slides were exposed for four weeks under NTB-2 autoradiographic emulsion (Kodak).

Fusion protein and immunisation: The full-length clone obtained from the CSML-100 λ phage-cDNA library was excised using EcoRI and ligated into pGEX-30X containing an in frame EcoRI-site under the control of the lacZ-promoter. The correct orientation was confirmed using XhoI restriction site analysis. Expression of fusion protein was induced with 0.1mM IPTG, when the OD₆₀₀ reached 0.5. Harvested bacteria were resuspended in 10 mL STE (10mM TRIS pH=8.0, 150mM NaCl, 1mM EDTA) supplemented with 1mg lysozyme. After 15 min 5mM DTT, 0.2mM PMSF, 1.5% N-laurylsarcosine were added and the bacteria sonicated for approximately 3x20s. The fusion protein was purified on a glutathione column (Pharmacia, Sweden), washed and eluted with 20mM glutathione according to the manufacturer's instructions. Before immunisation the fusion protein was dialysed against PBS. Three rats were used for immunisation. Each animal was injected sc four times in all, once every other week, with 100 μ g/rat/immunisation. The first immunisation was supplemented with Freund's incomplete adjuvans.

Immunohistology: 2×10^5 CSML-0 or CSML-100 cells were injected sc into 10-week-old mice. 8-12 weeks later the tumour was excised, fixed in 4% formaldehyde, embedded in paraffin and slides were prepared. The paraffin-embedded tissue were rehydrated, microwaved in 0.01 M Citrate pH 6.0 for 2 x 5 min, and incubated in 1% H₂O₂. Primary antibody incubation was done for 60 min using anti-cystatin C polyclonal antibodies diluted 1:200 in 10% foetal calf serum. Secondary antibody goat-anti-rabbit (P448, DAKO, Denmark) was diluted 1:100 in 5% normal mouse serum and incubated for 30 min. After incubation the slides were rinsed and coloured with diaminobenzidine for 10 min.

Immunocytochemical staining: Cells were washed with PBS and fixed with 1:1 methanol:acetone. Primary (anti-E30 serum) and secondary (horseradish peroxidase labelled goat anti-rat, DAKO, Denmark) antibody were added sequentially for 1 hour in

PBS plus 10% FCS. Colour was developed with 3-amino-9-ethylcarbazol (Sigma, USA) in 15mM HAc, 35mM NaAc and H₂O₂.

Protein localisation: This method was adapted from (148). One 10 cm dish 100% confluent CSML-100 cells (app. 1×10^7 cells) were washed three times in PBS without calcium and magnesium, harvested mechanically and pelleted. The pellet was dissolved in 250 μ L buffer A (20mM HEPES (pH=7.2), 50mM KCl, 3mM MgCl₂ and 0.25M sucrose) and mixed with 250 μ L buffer A supplemented with 0.2% Triton X-100, incubated one minute at room temperature and pelleted at 316g for four minutes. The Triton X-100 soluble fraction was saved (the membrane and cytosol fraction), the pellet resuspended in 250 μ L buffer B (20mM HEPES (pH=7.2), 50mM KCl, 3mM MgCl₂ and 1mM CaCl₂), mixed with 250 μ L buffer B supplemented with 0.2% NP-40 and homogenised for 5 minutes. The solution was pelleted for 5 minutes at 700g resulting in the NP-40 soluble fraction (the cytoskeletal fraction) and the pellet (the nuclear fraction). The three samples were tested in a Western blot as described.

Transfection: CSML-0 or CSML-100 cells were cultured in DMEM supplemented with 10% FCS. Cells were collected after trypsin-EDTA treatment, washed twice with PBS and used for electroporation. For each transfection 100 μ L containing $1-2 \times 10^6$ cells and 10 μ g pRc/CMV-10 or pRc/CMV-11, carrying the neomycin resistance gene, were electroporated at 250V and 250 μ Fd using Bio-Rad electroporation system. Cells were plated directly in 5 96-well dishes. Sixteen hours after electroporation selection of transfected cells with G418 was commenced and continued for three weeks.

3. Results

Cloning genes differentially expressed in metastatic and non-metastatic tumour cell lines

Three mammary adenocarcinoma cell lines were used in DD to identify genes putatively involved in the metastatic process. They were CSML-100 (metastatic to the lungs), CSML-0 (originating from the same tumour as CSML-100, but non-metastatic) (136) and VMR-Li (metastatic to the liver and lungs) (137). For the DD

we used 32 upstream arbitrary 10-mer primers (Table 2.1) and one anchoring primer (T₁₁AG). The reactions were repeated (reverse transcription and PCR) and reproducible bands differing in expression among the cell lines, were excised. Patterns of gene fragments were largely identical among the three cell lines (Fig. 3.1). The strands of the amplified DD-fragments were separated on the denaturing sequencing gel resulting in several bands on the gel. The appearance of these bands was explained partly by the tendency of the taq-polymerase to add an extra adenosine to the 3' end of the PCR-product and partly by differences in molecular weight and mobility in the gel of the strands. When a non-denaturing gel was used these multiple bands from the same amplification product were eliminated (data not shown). The separation of strands was

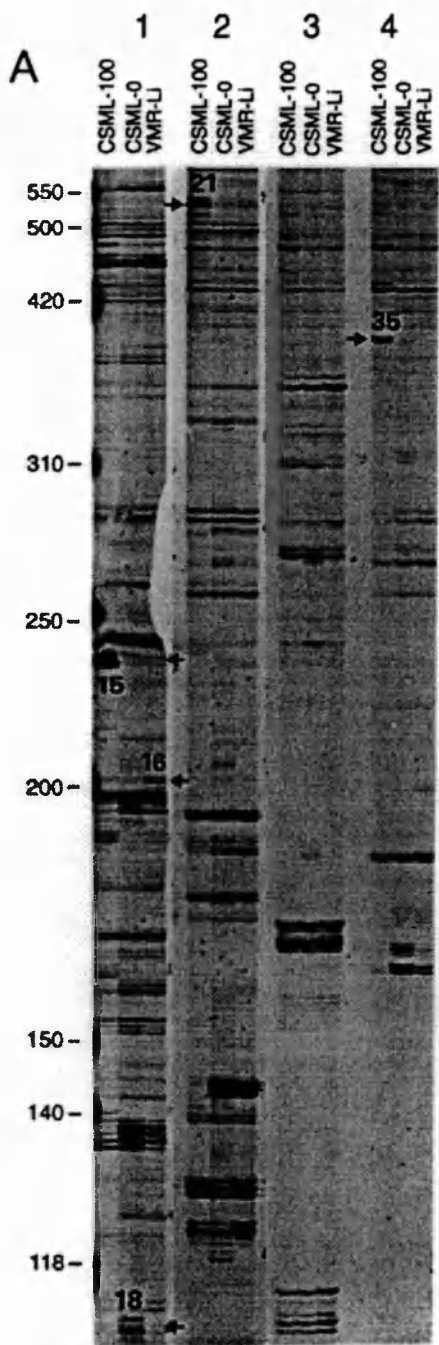


Figure 3. 1: Identification of cDNA-fragments differentially expressed in DD in metastatic versus non-metastatic cell lines. Shown are the DDs carried out using downstream primer CAT₁₁, and upstream primer 8, 9, 13 and 14 in lanes 1-4 respectively. The candidate cDNA-fragments (numbered), reproducibly differentially displayed, were excised.

more pronounced for the smaller fragments in the better separating part of the gel. A total of 56 fragments were selected for further analysis. The selected fragments were 100% successfully PCR-amplified using the same conditions as used during the DD (Fig. 3.2). The sizes of the selected DD-fragments ranged from 90-600 bp and corresponded to the sizes of the PCR-fragments. Except for fragment 50 a single band was amplified in the preceding PCR of the fragments extracted from the gels. The upper band of fragment 50 corresponded in size to the excised fragment.

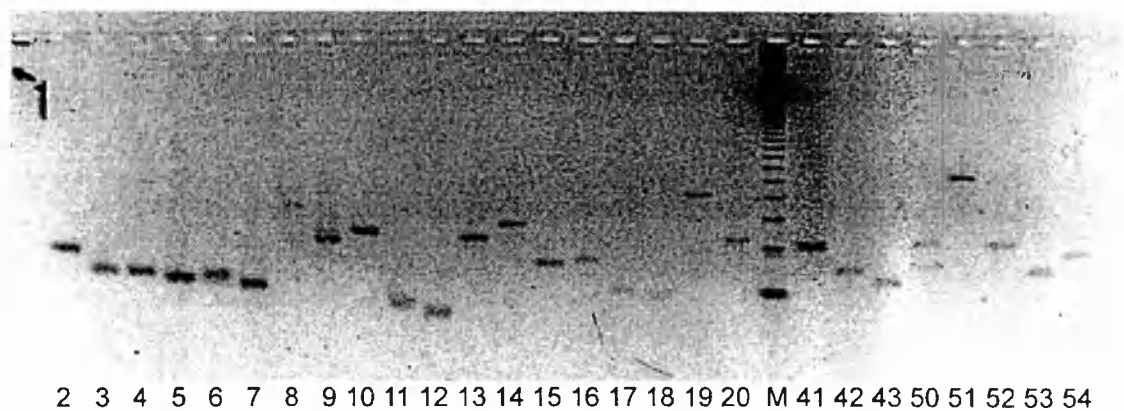


Figure 3.2: PCR-amplification of DD-fragments. A selection of fragments excised from the sequencing gel. The fragments (number of fragment stated below the gel) were eluted from the sequencing gel, PCR amplified under the conditions used during the DD and separated on a 2% agarose gel. The marker (M) is a 110 bp ladder. The picture is shown as a negative to facilitate printing and improve visibility.

The amplified fragments were labelled by either PCR or random priming and used as probes for Northern hybridisation. At this stage, 31 of 56 fragments were selected for further analysis (Fig. 3.3 and Table 3.1). While differential expression in some instances was obvious (i.e. fragment 2, 3, 4, 10, 15, 18, 19, 21, 23, 30, 32, 42, 44, 46, 51), others were more subtle (i.e. fragment 5, 7, 9, 13, 14, 31, 35, 54 and 55).

Evaluation of the results of the Northern blot hybridisation was complicated by occurrence of several bands in each hybridisation. Over exposure of a DD-gel revealed radioactivity on all positions in the gel. It would therefore be surprising if the excised fragments were pure. These impurities thus resulted in background hybridisations obscuring identification of differentially expressed genes. Only three of the selected fragments hybridised with low or no background detecting differences in gene expression (Fig. 3.3: fragments 18, 21 and 42), 28 were selected in spite of a background signal (Fig. 3.3, the rest), 10 failed to show any signal, while there was no difference in expression among the three cell lines for the remaining 15 fragments (data not shown).

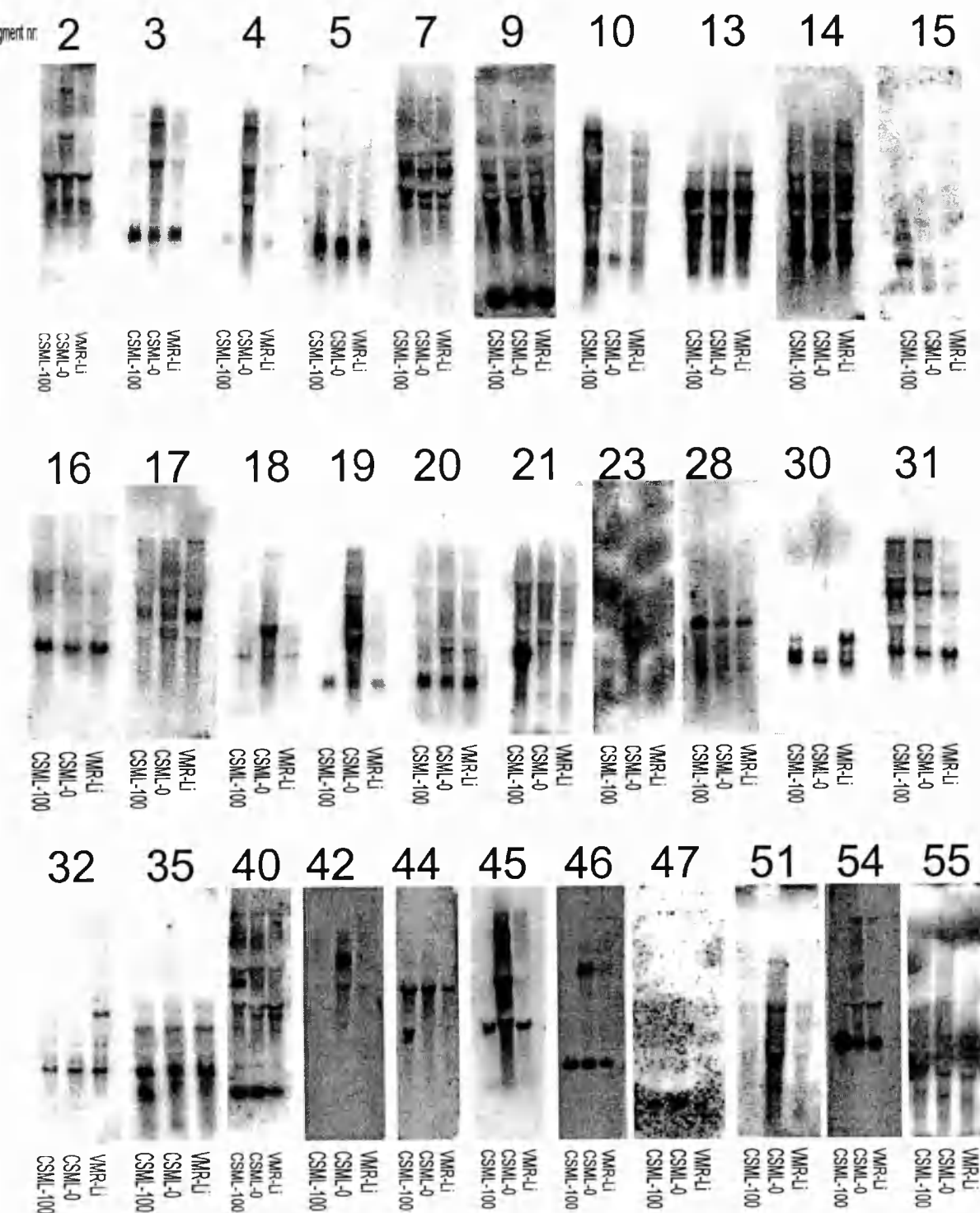


Figure 3.3: Secondary screening of 31 selected and reamplified fragments against totalRNA from CSML-100, VMR-Li and CSML-0. Of the 56 excised fragments differential display 31 of these could be detected in Northern blotting with a possible differential expression. App. 10 µg of RNA from the specified cells were loaded pr lane.

The 31 fragments selected on the basis of the initial Northern blot hybridisations, were cloned, labelled and used in a second round of Northern blot hybridisation (Fig. 3.4). The positive clones (from 95 to 536 bp long) were sequenced and a search for homology was made in the European Molecular Biology Laboratory (EMBL) database using the FASTA and/or BLAST sequence alignment programs (homologies are stated in Table 3.1). The pattern of expression found in the primary Northern blots generally corresponded to the pattern found in the cloned fragments in the secondary Northern blots. However, the Lamin C homologous clone 15 had an expression pattern not detected in the primary Northern blots of these two fragments (Fig. 3.3 and Fig. 3.4). This difference could be explained by for example impurities in the band excised, aerosol mixing of samples before PCR-reactions, overexpression of competing signals in the primary Northern blot hybridisation leading to cloning of an otherwise undetected gene fragment. Also, the expression patterns of the isolated clone 32 were unanticipated. In some instances (fragment 17, 23 and 44) a differentially expressed gene was convincingly detected in the primary Northern blot, but the corresponding gene fragment never cloned.

The cloned fragments were tested in Northern blot analysis against CSML-0, CSML-100 and VMR-Li. The potentially non-metastasis related genes collagen XI α 1, NN18 (clone18-4), MMTV and PACE4 were found mainly in CSML-0 cells, with small amounts of MMTV and PACE4 in VMR-Li cells (Fig. 3.4). The potentially metastasis related genes Cystatin C, Lamin C, Ly-6A/E, NN9 and NN32 were found in CSML-100 cells. While Ly-6A/E expression was confined to CSML-100, Cystatin C expression was also seen in CSML-0. Lamin C, NN9 and NN32 were overexpressed in the two metastatic cell lines with low amounts detected in CSML-0 (Fig. 3.4). Though the GAPDH labelling of the filter used for clone 21 (Ly-6A/E) showed, that it was not properly balanced, these data were confirmed (Fig. 3.6). The data for the cloning of fragment 30 is presented later.

The identified sequences were aligned with homologous sequences using Bestfit or Gap from the GCG-package (Fig. 3.5, mismatches depicted in reverse colours). We found that of 17 analysed fragments, five contained sequences corresponding to MMTV, four corresponded to the Lamin C gene, four were homologous to other

known genes (PACE4, Cystatin C, Collagen XI a1 and Ly-6A/E), three were homologous to previously published expressed sequence tags (ESTs) and one novel

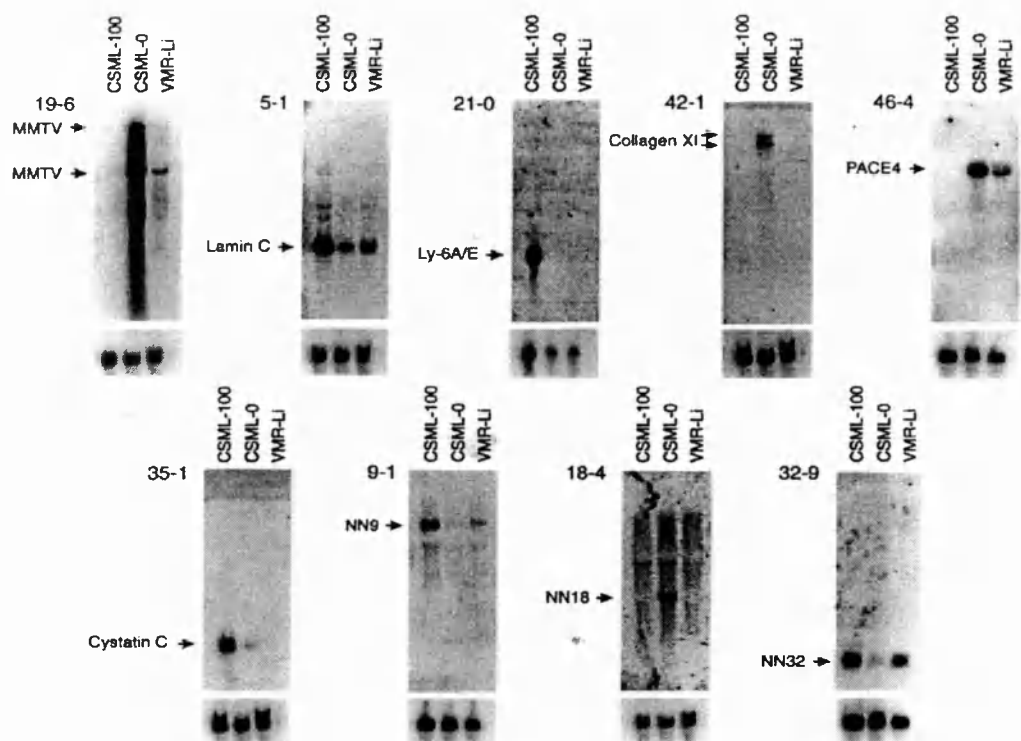


Figure 3.4: Nine different fragments displaying differential expression were cloned. The nine fragments with a reconfirmed differential expression pattern as shown in Northern blotting. Below each filter a corresponding GAPDH hybridisation as control of RNA loading. App. 10 µg of RNA was loaded pr lane. Sequencing and EMBL database searching revealed sequence homology to published genes. Arrows depicts differentially expressed genes indicating the possible homology.

DNA-fragment (Fig. 3.5 and Table 3.1) (data for fragment 30 is shown later). The EST-fragments, recognised by clone 18-4 and clone 9-1, were isolated from brain and mammary gland, respectively. Ignoring the mismatches in the primer regions and the previously unpublished 3'-stretches, four of the clones (clone 5-1, 21-o, 42-1 and 9-1) completely matched their homologue, while two clones (clone 35-1 and 46-4) contained a single mismatch. Mismatches in clone 19-1 (MMTV) recognising the 5' long terminal repeat probably arose from comparing different substrains of the virus, while clone 46-4 (PACE4) and clone 18-4 (NN18) were aligned to rat sequences. Due to the low fidelity of the taq-polymerase mismatches could also occur during PCR. All bands were found to contain the expected upstream and downstream primer sequence at the 5'- and 3'-ends underlined in figure 3.5. The 5'-primer site contained one or two mismatches compared with the known mouse sequence. However, in the

clone 19-6 (MMTV) Length: 492 bp
 1 TCGGTCATAG TACTTATGAT TTTCCCCCTT TTTTTCAGT GCTTTGCGAA GAGCCTTGAC CAAGTGCAGT CAGATCTTAA CGTGCT
 101 AAAAAAGGGG AAATGCCGCG CCTGCAGCAG AAATGGTGA ACTCCCGAGA GTGTCCTACA CTAGGAGAG AAGCAGCCAA GGGGTT
 201 ACGACCGTTC TGTGACAA CCGATGAGCC CATCAGACAA AGACATAATC ATTCTCTGCT GCAAACCTGG CATAGCTCTG CTTTGC
 301 GAAAGTTGGG GTTCGTGCTC GCAGGGCTCT CACCCTTGAT TCTTTTAATC ACTCTTCTGT GCAAGATTAC AATCTAAACG ATTCGG
 401 CTCTCTGGGC AAGGACCACA GCCAACTTCC TCTTAAGAG CCGGACTTTG TCCTTCAGAA ATAGAAATAA GAATGCTTGC TAAAAA

clone 5-1 (Lamin C) Length: 183 bp
 1 TACAAGGAGG ACAATGAGGA TGACGACGAG GATGGAGAAG AGCTCCTCCA TCACCACCGT GTGAGTGGCA GCCGCCGCTG AGCCCA
 101 CCCTGCCAGC CTAGGGCAGC TCTCCACCT CCATGCCAAA GTCTTTTCAT TAAAGAATGT TTTGGAATGC CTAATAAAAA AAA

clone 21-0 (Ly-6A/E) Length: 536 bp
 1 GGTACTAAGG TCAATGTGAA GACTTCCTGT TGCCAGGAAG ACCTCTGCAA TGCAGCAGTT CCCAATGGAG GCAGCACCTG GACCAT
 101 TGTTTACGCT GAGCTCAGTC CTCCTGCAGA CCTTGCTCTG ATGGTCTCTC CAATGACCTC CACCCTTGTC CTTTATCTCT CATGTG
 201 GGAGCCCTCT AGTGATGAAT TATGAGTTAT AGAAGCTCCA AGGTGGGAGT AGTGTGTGAA ATACCATGTT TTGCTTTAT AGCCCT
 301 TGCTCTAATC CTCTCTAGGG CTTTCAAGTC TGTACTTCT AGAATGTCAT TTTGTTGTGG ATTGCTGCTC ATGACCCTGG AGGCAC
 401 GAAGAGGCAG AATTCCAAGG TATTATGCTA TCACCATCCA CACATAAGTA TCTGGGGTCC TGCAATGTTC CCACATGTAT CTTGAA
 501 GTCCAATAAA CCCTTTGTTC TCCCTAAAAA AAAAAA

clone 42-1 (Collagen XI alpha1) Length: 178 bp
 1 ATCTAACC GAGAGGATTA TGATGTCCAG AGAAACACTT CCGAGGATAT TCTGTACGGG AACAAAGGAG TAGACGGCAG GGATTC
 101 ATGGAGATTG AGGTGAATAT GATTTTATG AATACAAAGA ATATGAAGAA AGGACTACGA CCTCCCCTAA TAAAAAA

clone 46-4 (PACE4) Length: 156 bp
 1 ATCTGA TAATGTGTTT GTCTTGTCAC CTGTAAGTGG CAAGGACTCA GCTGTTGTCT GTTGAATCCA CACTTCAAAT AAGAAA
 101 CTAATGTTAA CCCTGAGTAA ATAAAAAGG TAAAAAGGCTGAAAAA TAAAAA

clone 35-1 (Cystatin C) Length: 393 bp
 1 ATCAAGTCC CAGACAAATT TGACTGACTG TCCTTTCCAT GACCAGCCCC ATCTGATGAG GAAGGCACTC TGCCTCTCC AGATCT
 101 AAAGGCACAC ACTCCCTGAC AAAATTGAGC TGCAAAAATG CCTAAGGGCT GAGTCTAGAA GGATCATGCA GATTGTTCTT TACTTG
 201 AGTGTTTCAT CTCGCAGAAG GGTGCTCCGG CTCTGGAGGG CACCGCCAGT GTGTTTGAC CAGGAGACAG TAAAGGAGCT GCTGCA
 301 ATCTGAACAG CTGTCCCCTG GCTCCACTCT TCTTGCACTA CCTATCATGC CTGCTCAAT TAAAAA AAAA ACTCCTTTGG CTAAAA

clone 9-1 (NN9) Length: 309
 1 TTTCTACCC AGGAGATATC TTATATTGG AAAATGTCTG CCTCTCCAAA TAAATATGTG ATTAATAAGC CATATCAGAG TTTAAG
 101 CCCCTCCAAA AACCAAGTAT TTTGCATATG ATTGATTITA GAAAGATTTT GAAGCTGGGG TTTATCTGTG TAATGAAGAC CAGATT
 201 TGCAATTGTA ATTTTCAAAT GAAATTTCTT ATACGATTAT TAAAAAAGT GTTGTGTTTC CTGTGTTT ATTAATAAAT TAAAAA
 301 TAAAAAAT

clone 18-4 (NN18) Length: 111
 1 CTAAGG CAAAAAGGGT AGGGCCAGGT GTTCTGTCT TGGGAATGTA GGGCTATCCT TACTGTGTA AATAAACCTT TAAATG
 101 TAAAAAAT

clone 32-9 (NN32) Length: 95
 1 AGCTACTGAC ACACATGAAC TTTACACAC AAATAAATGA CTATATATGA TAAAAATTAAG GAGAAAAAAA TTCTCCCTTG TGCTAA

Figure 3.5: Genes identified using DD from metastatic or non-metastatic tumour cell lines. Nucleotide sequence of the 9 differentially expressed fragments. Clones homologous to sequences in the EMBL-database were compared to corresponding known genes. The comparison revealed several mismatches, deletions or unknown 3'-sequences indicated in reverse colours. Primer sites are underlined, the box depicts an upstream primer site in Lamin C better than the one used. Sequences were compared to other mouse sequences except clone 46-4 and 18-4 compared to rat sequences.

Lamin C sequence the 5'-primer detected a site of only four matching nucleotides. Curiously, though the PCR technique has a tendency to favour shorter amplification products, the Lamin C sequence contained a better primer recognition site, 21 bases downstream of the utilised primer site (Fig. 3.5, boxed, 8 matching nucleotides). None of the 3'-end sequences in the EMBL database matched the complement of the downstream primer sequence CTA₁₁ found in the cloned fragments. This variation in site of polyadenylation could indicate that the DD-selected 1/12 of the transcripts did not represent 1/12 of the transcribed genes. The fragments homologous to the MMTV gene and the Collagen XI α 1 gene corresponded to an internal part of the transcript corresponding to a 5'-CTAAAAATTATAT-3' and a 5'-CTAATGAAGAATT-3' stretch inside the RNA, respectively.

Table 3.1:*The 31 selected fragments differentially expressed in the CSML cell line system.*

Fragment #	Primer Up	Fragment Size	DD Expression	Clones w/ diff. expr.	Homology ^{a)}	Approx. size of transcript
2	1	250 bp	CSML-0	+	MMTV	10 kb; 4.5 kb
3	1	200 bp	CSML-0	+	MMTV	10 kb; 4.5 kb
4	1	190 bp	CSML-0	+	MMTV	10 kb; 4.5 kb
5	1	180 bp	CSML-100/VMR-Li	+	Lamin C	2.0 kb
7	1	160 bp	CSML-100			
9	3	300 bp	CSML-100	+	EST(AA427021)	4.5 kb
10	4	320 bp	CSML-0/VMR-Li			
13	6	300 bp	CSML-0			
14	8	400 bp	CSML-0/VMR-Li			
15	8	240 bp	CSML-100	+	Lamin C	2.0 kb
16	8	200 bp	VMR-Li	+	Lamin C	2.0 kb
17	8	120 bp	CSML-0			
18	8	110 bp	CSML-0	+	EST(W71785)	2.0 kb
19	9	450 bp	CSML-0	+	MMTV	10 kb; 4.5 kb
20	9	250 bp	CSML-0			
21	10	500 bp	CSML-100	+	Ly-6A/E	0.9 kb
23	10	115 bp	CSML-0			
28	E	190 bp	CSML-100/VMR-Li	+	Lamin C	2.0 kb
30	E	118 bp	CSML-100/VMR-Li	+	E30 ^{b)}	1.4 kb
31	E	100 bp	CSML-0/VMR-Li			
32	E	90 bp	CSML-100	+	No match	0.5 kb
35	14	400 bp	CSML-100	+	Cystatin C	0.7 kb
42	21	180 bp	CSML-0	+	Collagen XI α 1	7.0 kb
44	21	100 bp	CSML-100			
45	23	310 bp	CSML-0	+	MMTV	10 kb; 4.5 kb
46	23	150 bp	CSML-0	+	Rat PACE4	4.2 kb
47	23	110 bp	CSML-0			
51	AP2	600 bp	CSML-0			
54	AP3	220 bp	CSML-0			
55	24	400 bp	CSML-0			

^{a)} The protein names of the homologous sequences are mentioned. Sequence data are from mouse except rat PACE4 and rat EST(W71785).

^{b)} A new gene described here.

Expression in mouse cell lines with varying metastatic potential

To further investigate the possible significance of the isolated genes in tumour progression, we compared transcription of six of the genes to the expression pattern of known metastasis-related gene (i.e. E-cadherin) using a panel of 14 mouse cell lines of varying tumourigenic and metastatic potential. The genes identified in this study, but not tested in this panel were: MMTV, since its involvement in progression is believed to be secondary, possibly via activation of non-MMTV related genes during

integration into the genome (149), and clone 18-4, since the CSML-0 hybridisation from this clone faded during the experiment. Trying to hybridise this clone to the panel of cell lines gave no signals. The panel of cells consisted of four related adenocarcinoma cell line systems, the previously mentioned VMR and CSML, plus MT1 (138) and RAC (139) systems and three additional cell lines, Line1(141), LL-

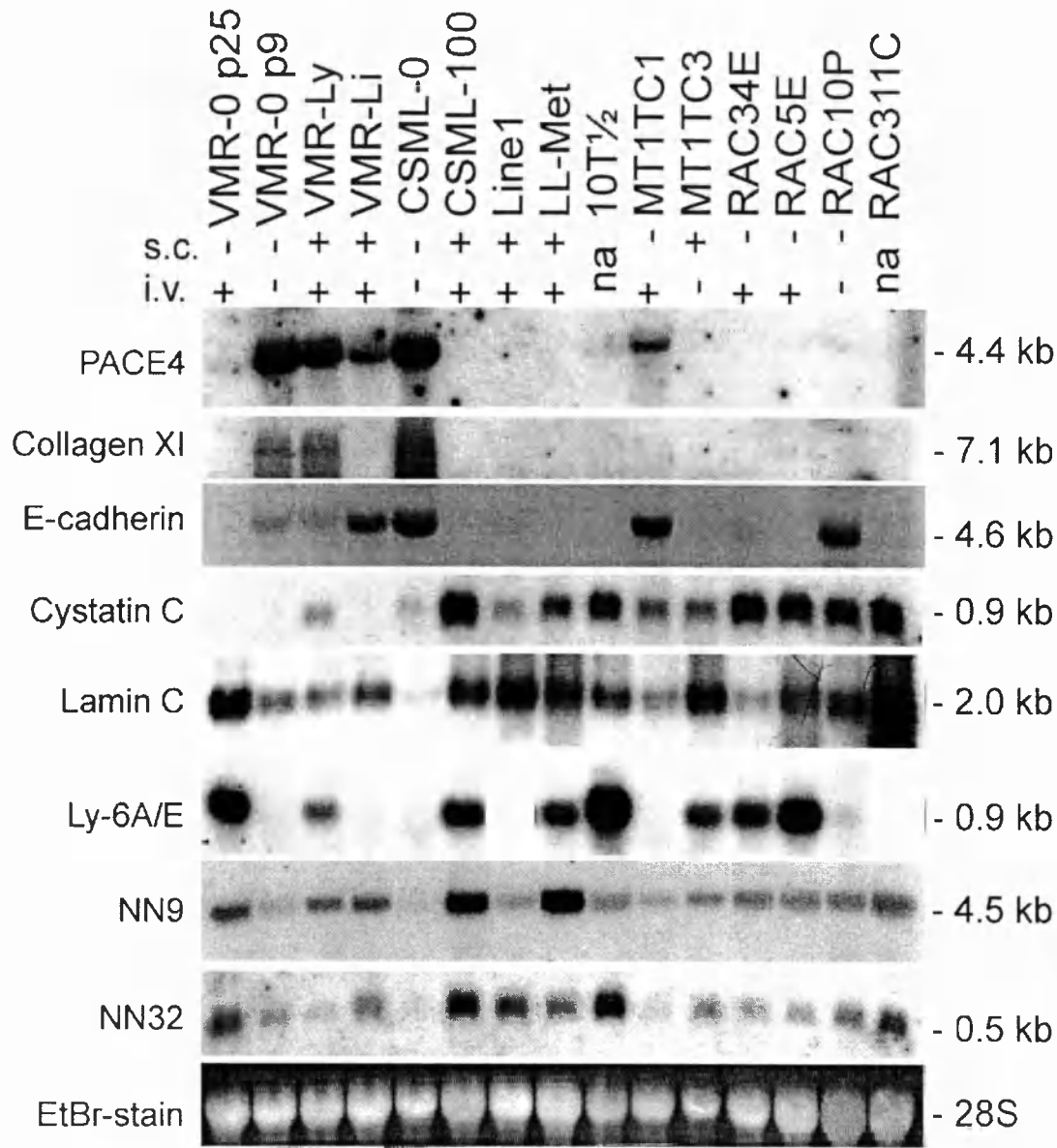


Figure 3.6: Northern blot analysis of probes corresponding to PACE4, Collagen XI α 1, E-cadherin, Cystatin C, Lamin C, Ly-6A/E, clone 9-1 and clone 32-9 in a panel of 14 mouse tumour cell lines. Metastatic (+), non-metastatic (-) or not assayed (na) in an assay using either subcutaneous (s.c.) or intravenous (i.v.) injections (151). Sufficient amounts of RNA was prepared and adjusted according to (120) and used for loading several gels with 10 μ g per lane. A sample EtBr-stained gel is shown.

Met (142) and 10T $\frac{1}{2}$ (143). The panel contained two non-tumourigenic cell lines (10T $\frac{1}{2}$ and RAC311C), three non-metastatic cell lines (VMR-0 p9, CSML-0 and RAC10P), one cell line metastatic in a spontaneous assay only (MT1TC3), four cell

lines displaying metastatic behaviour in the experimental assay only (VMR-0 p25, MT1TC1, RAC34E and RAC5E) and five metastatic cell lines metastatic in both assays (VMR-Ly, VMR-Li, CSML-100, Line1 and LL-Met). The VMR-0 cells became experimentally metastatic when passaged from p9 to p25.

As can be seen, expression of PACE4 mRNA closely resembles the expression pattern of E-cadherin since VMR-0 p9, VMR-Ly, VMR-Li, CSML-0 and MT1TC1 all express both genes (Fig. 3.6). There are two discrepancies: E-cadherin is expressed in RAC10P, while barely detectable amounts of PACE4 mRNA are found in 10T½. The gene coding for Collagen XI α 1 is transcribed only in the E-cadherin expressing cell lines, VMR-0 p9, VMR-Ly and CSML-0, in the latter in an alternatively spliced form as shown (Fig. 3.4 and 3.6) (150).

The genes upregulated in CSML-100 cells were transcribed in most of the cell lines. The transcription of the gene coding for Cystatin C was suppressed in VMR-0 p9, VMR-0 p25, VMR-Li and CSML-0 and present in all other cell lines. In the VMR cell system expression of Cystatin C was elevated in the VMR-Ly, in the CSML cell system CSML-100 expression was increased, it was upregulated in the highly metastatic cell lines Line 1 and LL-Met, while no variation in expression could be detected in the MT1 and RAC cell line systems.

Transcription of the gene encoding Lamin C was found in all the cell lines at variable strength. However, it was upregulated in cell lines metastatic in the spontaneous assay and in VMR-0 p25.

Ly-6A/E expression was confined to metastatic cell lines, in that VMR-0 p25, VMR-Ly, CSML-100, LL-Met, MT1TC3, RAC5E and RAC34E express and the non-metastatic CSML-0, VMR-0 p9, and RAC10P do not. Lack of expression in Line1 was shown elsewhere (152).

The two unknown genes also detected transcripts in all cell lines. Increased expression of NN9 transcripts was found in the metastatic cell lines of the VMR and CSML cell line systems, and with strong expression in LL-Met cells. NN32 displayed

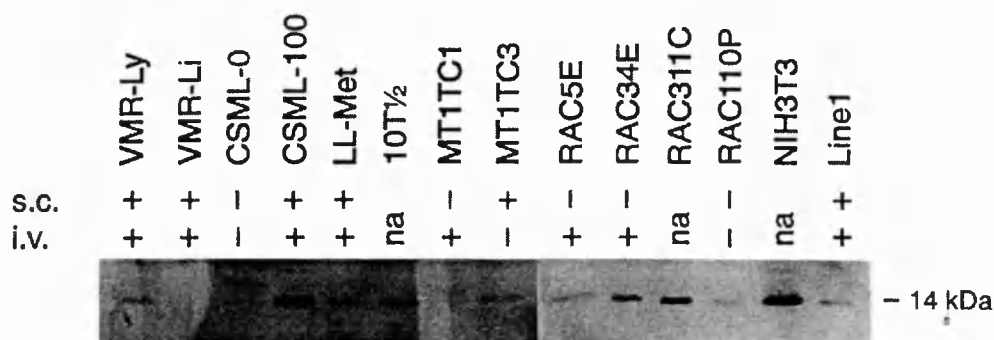
increased expression in most of the cells capable of spontaneous metastasis to the lungs, i.e. VMR-Li, CSML-100, Line1, LL-Met and MT1TC3. Also, expression was elevated in the non-tumourigenic 10T½ and RAC311C. An attempt was made to isolate full-length NN32 from a CSML-100 cDNA library. However, all twelve potential full-length clones obtained had no homology to NN32. Instead they were homologous to galectin-1 (data not shown).

In general the genes upregulated in CSML-0 had a tendency to be expressed in VMR-0 p9 and MT1TC1, and not expressed in VMR-0 p25, CSML-100 and MT1TC3. As opposed to this expression pattern, the genes upregulated in CSML-100 tended to be expressed in Line1, LL-Met and VMR-0 p25 as well as in the two non-tumourigenic cell lines, but downregulated in CSML-0 and VMR-0 p9. Only in the case of Ly-6A/E did the RAC cell lines show differential expression of an identified gene.

Analysis of Cystatin C by Western blot and immunohistology

Since, the expression of Cystatin C in Northern blots was seen to correlate with the metastatic phenotype, the correlation of expression of protein using Western blot analysis and immunohistology was analysed. Cystatin C was detected in *in vitro* culture of CSML-100 cell lysates with the expected size of 14kDa, while the CSML-0 cells displayed only low amounts of the protein (Fig. 3.7A). Basically the protein expression corresponded to the level of mRNA detected, but lower than expected protein levels were seen in the MT1TC1, RAC5E and RAC10P cell lines. Immunohistological examination of Cystatin C expression in tumours originating from CSML-0 and CSML-100 cells revealed increased overall staining of Cystatin C in the CSML-100 tumour as compared with the CSML-0 tumour (Fig. 3.7B).

A



B

CSML-0

CSML-100

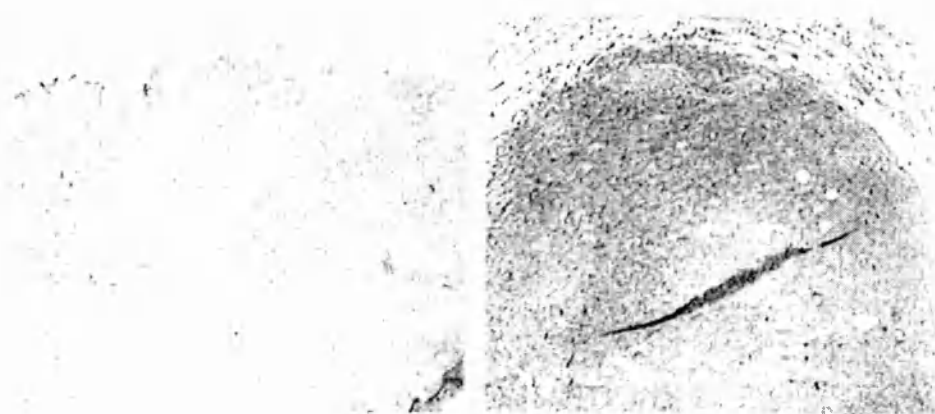


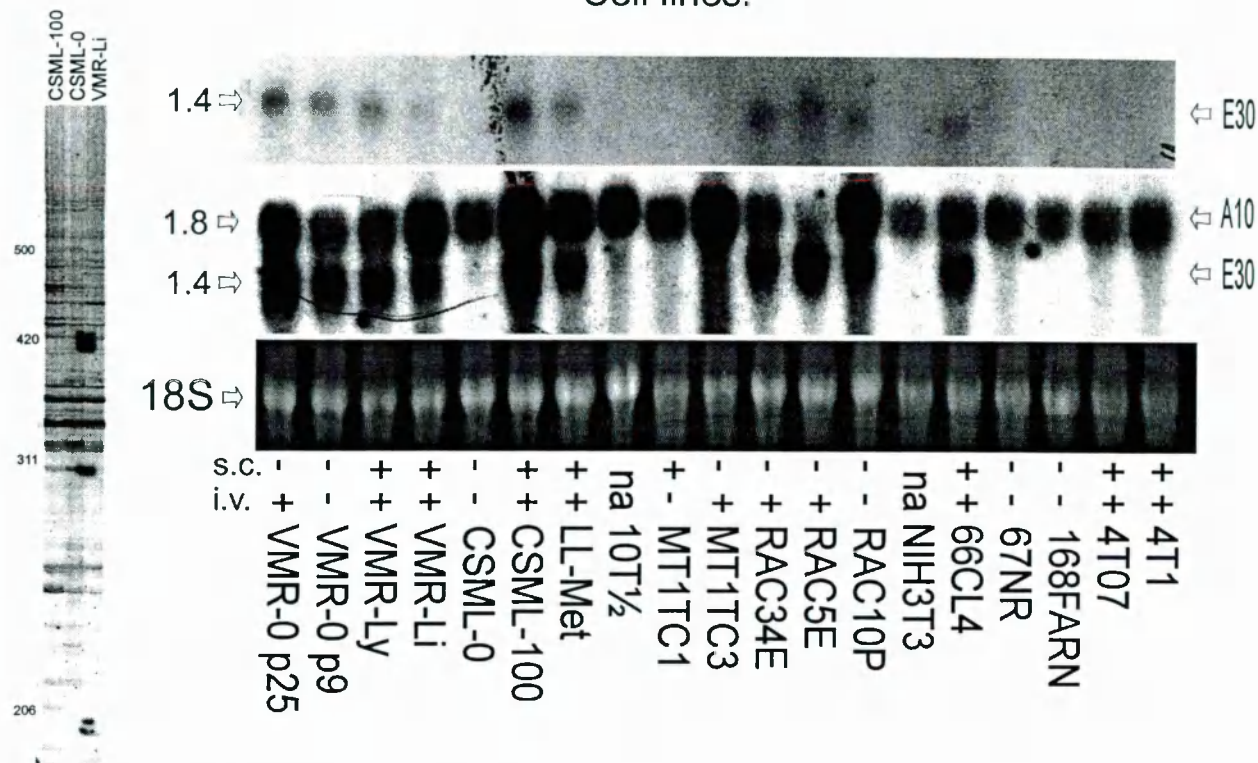
Figure 3.7: Distribution of Cystatin C protein in cell lines and tumour tissue. (A) Detection of cystatin C in 14 cells lines of different metastatic potential by Western blot analysis. Total lysate of app. 10,000 cells were separated on a 15% SDS-PAGE gel, blotted, labelled and stained using ECL. Ponceau S staining of the filter was used to control the amounts of protein blotted to the membrane. Abbreviations as in Fig. 3.3. (B) Immunohistology using anti-cystatin C polyclonal antibody. Sections of tumour from metastatic CSML-100 cells and from non-metastatic CSML-0 cells were used for immunohistology. 40x times magnification shown. The tumour arising from metastatic cells reacts intensively with the antibody.

Due to the hybridisation pattern of clone 30-8 to CSML-100, VMR-Li and CSML-0 in Northern blot analysis further investigations of this clone were carried out.

A

C

Cell lines:



B

D

Embryo:

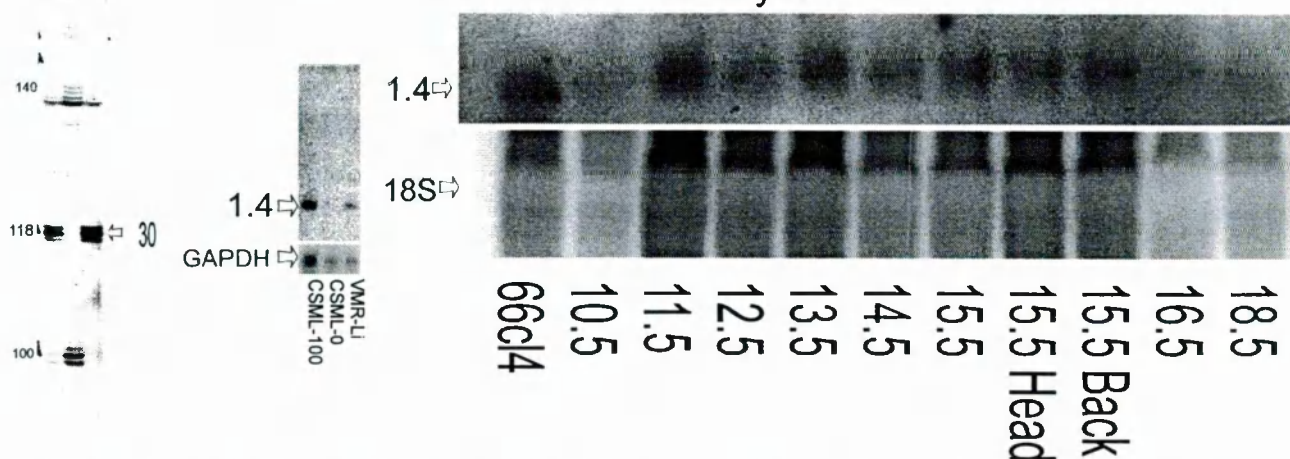


Figure 3.8: Identification and RNA expression of fragment E30. (A) A DD carried out using downstream primer CAT_n and upstream primer E. The fragments depicted by the arrow were excised. Size in bp is indicated to the left. (B) The fragments were cloned and retested showing differential expression in Northern. A GAPDH-hybridisation is shown below. (C) Transcription of E30 was tested in Northern against the panel of cell lines introduced in Fig. 3.6. The size of the E30 recognised transcript was app. 1.4 kb. The upper blot shows hybridisation with E30 alone. Below a stronger hybridisation also containing transcription factor A10 of 1.8 kb as control for size and presence of RNA. The 18S band of the EtBr-stained gel is shown below. (D) Northern blot of E30 hybridising to RNA from embryos. PolyU-hybridisation is shown below. E30 was detected at 10.5-18.5 dpc. App. 10µg/lane of total RNA were loaded.

Complete coding sequence of clone 30-8

Due to potential patenting rights the sequence is not stated in the text, which unfortunately makes description and review less practical. Please excuse the inconvenience.

Figure 3.8 depicts the isolation and RNA expression pattern of fragment 30. Fragment 30 was isolated in the same round of DD as described above. The 118 bp fragment was expressed in the two metastatic cell lines CSML-100 and VMR-Li (Fig. 3.8A). This expression pattern was confirmed by Northern blot analysis prior to and after cloning (Fig. 3.4 fragment 30 and Fig. 3.8B).

Northern blot revealed expression in several metastatic cell lines (Fig. 3.8C). Thirteen of the cell lines in the panel of cells presented previously (Fig. 3.6 and above) was supplemented with the Miller cells: 66CL4, 67NR, 168FARN, 4T07, 4T1 isolated from the same adenocarcinoma (140) and the non-tumourigenic, fibroblast cell line NIH3T3. The metastatic ability of the cell lines is depicted in figure 3.8C (151). A 1.4 kb transcript was found in the metastatic VMR cells, CSML-100, LL-Met, RAC34E, RAC5E and 66CL4, but not in the non-metastatic CSML-0, 67NR and 168FARN, and the non-tumourigenic NIH3T3 and 10T½ fibroblast cell lines. Though expression was found in non-metastatic RAC10P and VMR-0 p9 and no expression was found in the MT1 and 4T cell lines these data encouraged further investigations into the potential role of E30 in tumour progression.

To investigate expression of E30 during embryogenesis, Northern blot analysis was performed using total RNA from embryos of days 10.5 to 18.5 of pregnancy (Fig. 3.8D). The E30 transcript was detectable during a period extending from day 10.5 to day 18.5 of mouse embryogenesis. At stage 15.5 dpc transcripts were present in both the head and back of the embryo (Fig. 3.8D). The faint signals obtained at day 10.5, 16.5 and 18.5 was due to smaller amounts of RNA loaded as judged from the polyU hybridisation.

Sequence information

Sequencing and database analysis did not reveal relation to any complete coding sequence, but only to several ESTs. ESTs are short sequences obtained sequencing PCR-fragments from different cDNA-libraries with the purpose of mapping the

expressed genes in a given sample. The sequence and its origin are the only information available. The 3' end lacked a conventional polyadenylation signal like AATAAA or ATATAA (153). However, alternative sequences like TATAA, GATAA or AATTA resembling polyadenylation signals were all situated less than 25 bp from the non-poly-A part of the 3' end. The ends corresponded to primers used.

Expression in the embryo (Fig. 3.8D) and in uterus of pregnant mouse (data not shown) validated further analysis of E30, since specific cells in these organs (trophoblast invasion of the uterus and migration of mesenchymal cells during embryogenesis) are models for metastasis. In order to obtain the complete coding sequence, a λ -phage cDNA library derived from the metastatic CSML-100 cell line was screened using fragment 30. When screening 300.000 plaques, one clone was found with a 3' end matching fragment 30. This clone contained an 1137bp E30 fragment and 1700bp of 5' A10 transcription factor sequence separated by an EcoRI-

site. The A10 transcription factor was shown to be ubiquitously expressed in the mouse carcinoma cell lines (Fig. 3.8C). Data processing revealed one large open reading frame (orf) in frame 2 of the E30 sequence (Fig. 3.9A). This orf has a potential ATG translation initiation sites, one of which is situated at nucleotide 23 and ending with a stop codon at position 1036 (followed by two more within the next 12 nucleotides). This orf should then code for a protein of 338 aa corresponding to a calculated

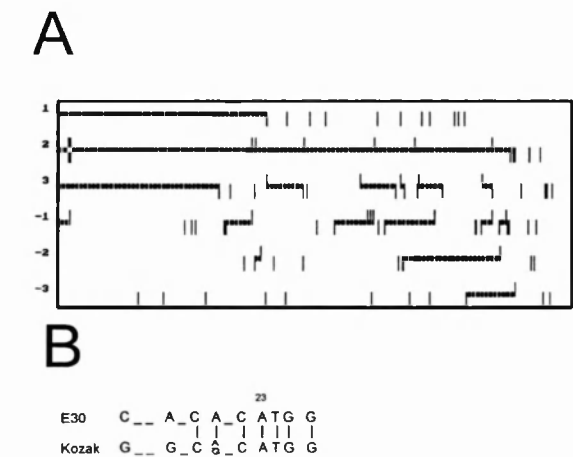


Figure 3.9: Orfs computed in the potentially full length cDNA-clone of E30. (A) One large orf in reading frame 2 of the cDNA was found with an initiating methionine coding ATG at position 23 and a terminating TGA at position 1036, potentially producing a 338 aa protein. There are several stop codons preceding this first stop codon. Line up and line down depicts methionine and stop codons, respectively. (B) The initiating ATG is surrounded by a sequence closely resembling the Kozak sequence predicting ATG-codon use. The significant nucleotides of the Kozak sequence are compared to the sequence of E30 with lines indicating homology.

molecular weight of about 39kDa. The translational initiation site conformed to the rules set out by Kozak (154), since seven out of nine important nucleotides match this sequence (Fig. 3.9B).

Structure of E30 protein

Analysis of the structure of the putative protein did not reveal any specific traits. For instance no obvious signal or transmembrane sequence was found in the Kyte & Doolittle hydropathy plot (Fig. 3.10A). The composition of the protein was consistent with a “normal” protein, except for approximately twice as many tryptophan residues (2.4% as opposed to 1.3% in an “average” protein) five of them situated within the initial 51 N-terminal amino acids. Since tryptophan often participates in ligand-receptor bindings this could indicate an adhesion function of the N-terminal region. The putative protein contained five potential phosphorylation sites, usable by Protein kinase C, six usable by Casein kinase 2 and two potential N-glycosylation sites. Testing the sequence for known protein motifs using the MOTIF command in the

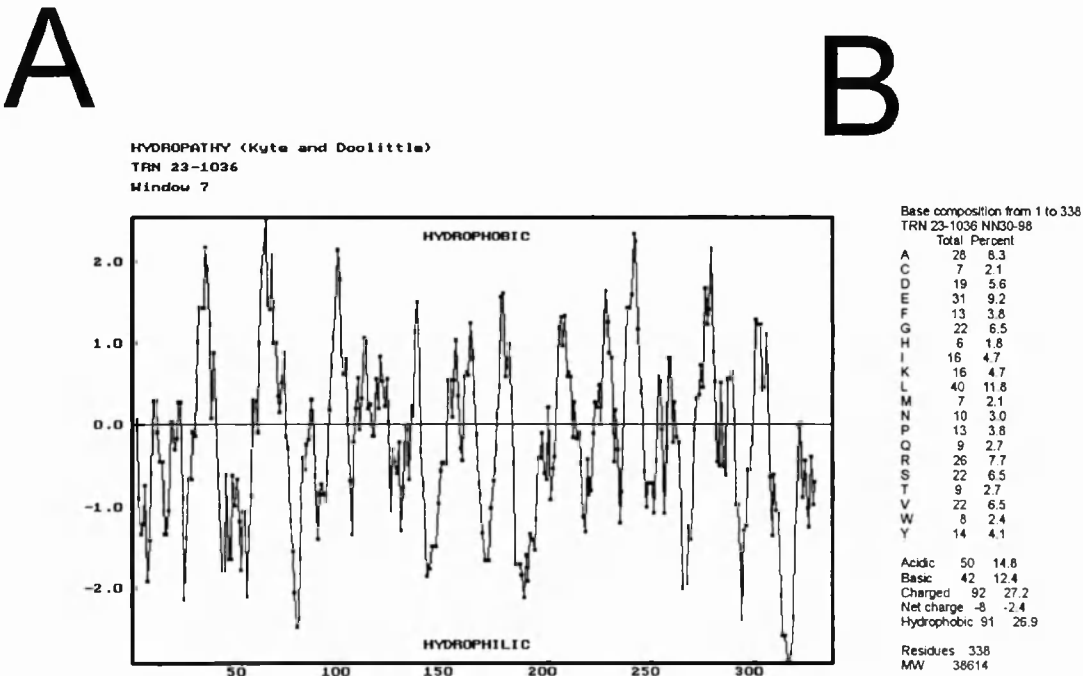


Figure 3.10: Hydropathy plot and composition of E30. (A) The hydrophobicity and hydrophilicity of E30 protein was calculated using the Kyte & Doolittle plot with a window of seven aas. No obvious transmembrane region or signal sequence was found. The composition of E30 protein was listed (B) revealing an “average” protein with only number of tryptophanes notably elevated. The theoretical molecular weight was calculated to be 38.6kDa and the net charge -8.

GCG package identified no additional known protein motifs. When allowing for one

mismatch, only six targets were identified, none making sense upon closer investigation (lacking a vital amino acid, positioned wrongly, prokaryotic motif only etc.). Allowing an additional mismatch did not reveal meaningful homologies either.

Homologies of E30 cDNA to mouse ESTs

A search for homologues of E30 was performed, comparing the sequence with sequences in several databases. No obvious homologies were found in the databases consisting of complete coding sequences. However, a number of EST fragments in the dbest-database displayed homology to E30 (Fig. 3.11). A group of mouse EST-fragments, almost identical to E30, was found in placenta, blastocyst, foetus at various days post coitum (dpc), T-cells, thymus, lymph nodes, mammary, kidney and heart, indicating expression of the gene in these tissue. This was in correspondence with the Northern blot of embryos at day 10.5 to day 18.5 (Fig. 3.8D).

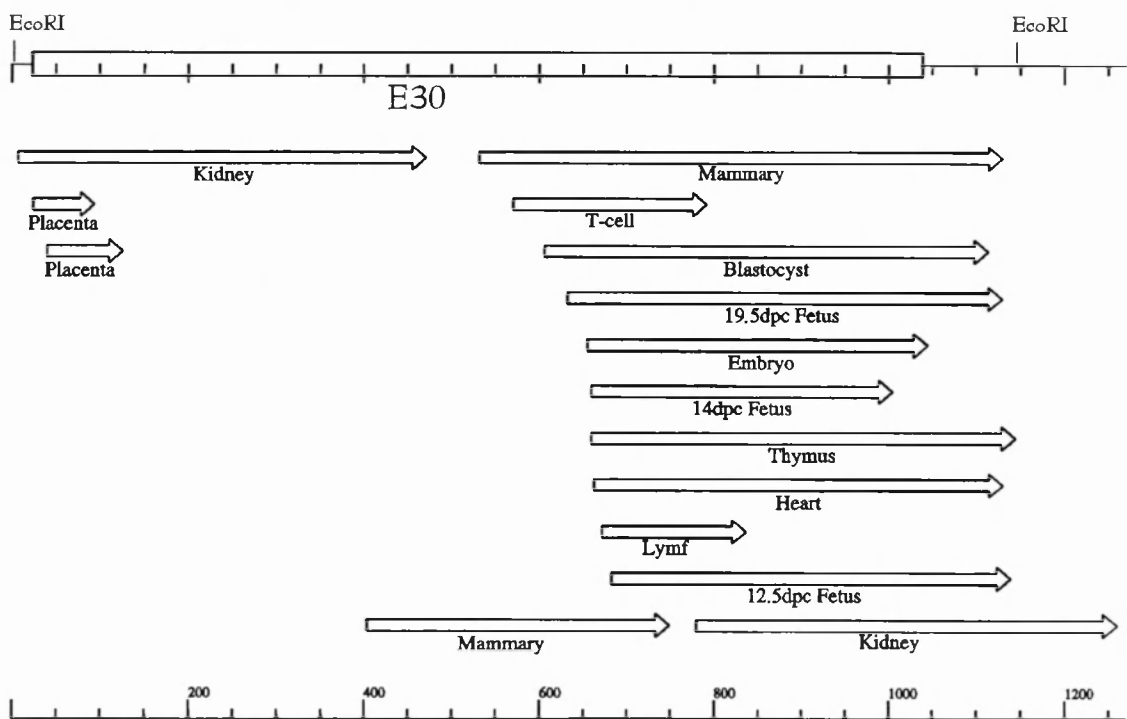


Figure 3.11: A representative selection of mouse EST fragments with homology to E30. A search in the dbest database revealed 23 DNA fragments > 99% identical to E30 from several different tissues covering the majority of the gene. The arrows depict the length of the fragments compared to E30 with the originating source indicated below. Dpc indicates days post coitum. The coding region of E30 is boxed.

Homologies of E30 cDNA to human and *C. elegans* ESTs

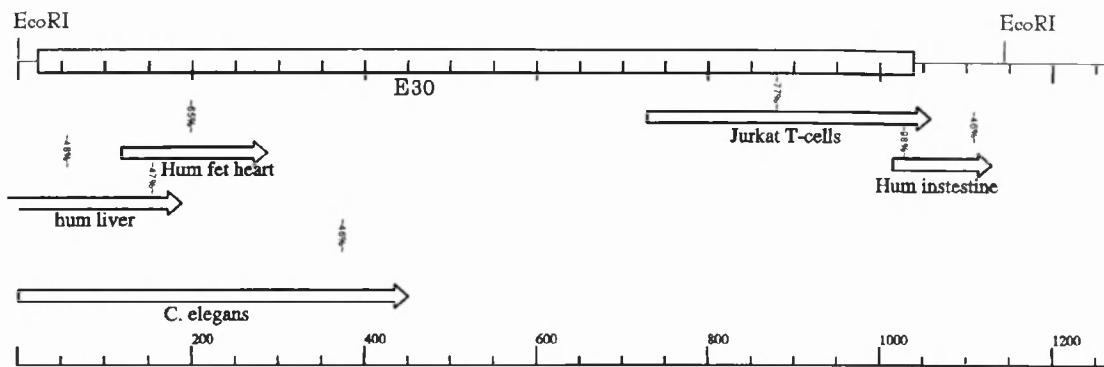


Figure 3.12: Non-murine ESTs. Five EST homologues isolated from human fetal heart (Hum fet heart), human liver (hum liver), human small intestine (Hum intestine), Jurkat T-cells or from *C. elegans* as revealed by searching the dbest-database. Symbols and layout are as in Figure 3.11. Relationship to E30 is in descending order (nearest homologues are closest to E30) and indicated as % identity at nucleotide level.

Also homologies to four human and a single *C. elegans* sequence were discovered, again in sequences from dbest (Fig. 3.12). The EST-fragment isolated from the Jurkat T-cell line was the closest relative being 77%/75% identical on the DNA level/amino acid level assuming the reading frame with the greatest matching score aligned using the GAP-program from the GCG-package. The remaining fragments were discovered in human small intestine (46%/85%), human foetal heart (65%/55%), human liver (48%/45%) and in *C. elegans* (46%/40%) with a DNA homology/protein homology as indicated in parenthesis. The high protein homology of the fragment from human small intestine is due to a highly identical short C-terminal homology of seven amino acids with only one mismatch, while the lower homology of the corresponding DNA sequence is due to the low pressure of conservation on untranslated 3' ends. The low homology of the putative protein from human liver can be improved to 54% (amino acid identity) if only the C-terminal 36aa were used for comparison. Whether the human homologous fragments represent one gene or several is not known. An attempt was made to isolate the DNA-sequence between the 5' end and 3' end fragments with an upstream primer matching the foetal heart fragment and a downstream primer matching the fragment from Jurkat. This attempt was unsuccessful (data not shown), a result which could be interpreted as pointing to the existence of more than one human gene matching E30. The overlapping fragments of EST-sequence from human small intestine and Jurkat cells are 98% identical (DNA). However, the overlapping fragments of EST-sequence from human liver compared to human foetal heart is only

47% identical on DNA-level and 59% on amino acid level providing more compelling evidence of the existence of more than one human gene homologous to E30.

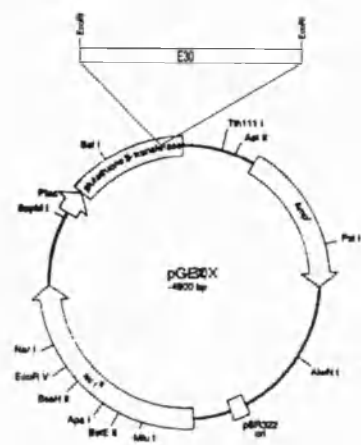
Cysteine and tryptophan residues are often conserved among species in homologous proteins. The potential protein contains seven cysteine residues (Fig. 3.10B), and when comparing with the non-murine sequences, four appeared conserved. The tryptophan residues were generally less conserved, with the only exception, a tryptophan in the 3aa-sequence SerPheTry as the most highly conserved sequence when comparing human, mouse and *C. elegans*.

Production of GST-E30 fusion protein

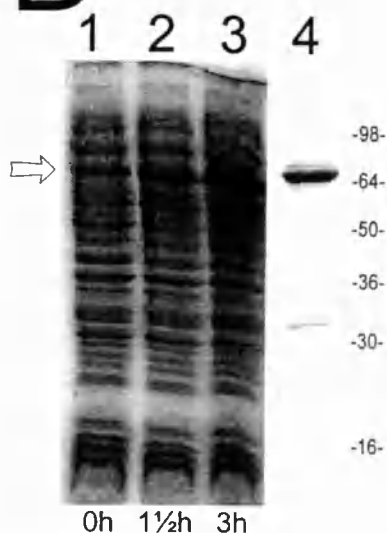
In order to produce protein for immunisation of rats, Western blot analysis and immunohistology, a fusion protein was constructed. The GST-system was selected due to the ease of purification of fusion protein with glutathione columns and experience for production of polyclonal antibodies within the institute. The E30 cDNA was placed into the pGEX-vector using an in frame EcoRI-site (Fig. 3.13A) resulting in an inducible fusion protein construct comprising of Glutathione-S-transferase N-terminally and E30 C-terminally spaced by 15 aa containing a Factor Xa site and seven aa arising from the otherwise non-coding 5' end of the E30 gene. Expression of fusion protein was even seen before induction as a band after SDS-PAGE. Induction of expression led to the highest expression after three hours (Fig. 3.13B). Though, extensive sonication was performed only approximately 50% of the protein was soluble. The purified fusion protein had a size close to the predicted 65kDa (Fig. 3.13B). Approximately 1.6mg was purified from a 500mL culture induced for 3 hours.

Three rats were immunised once, boosted four times, sacrificed and the sera tested for reactivity against E30 by Western blot analysis containing cell lysates from CSML-100, CSML-0 and VMR-Li (Fig. 3.13C). While the immune serum from rat 1816 showed reactivity against a protein of the expected approximately 39kDa, this reactivity was less pronounced than serum from rat 1817, and the serum of rat 1818 did not respond at all. Since the preimmune serum from 1817 contained reactivity against a protein of approximately 39kDa, the serum from rat 1816 was used in all

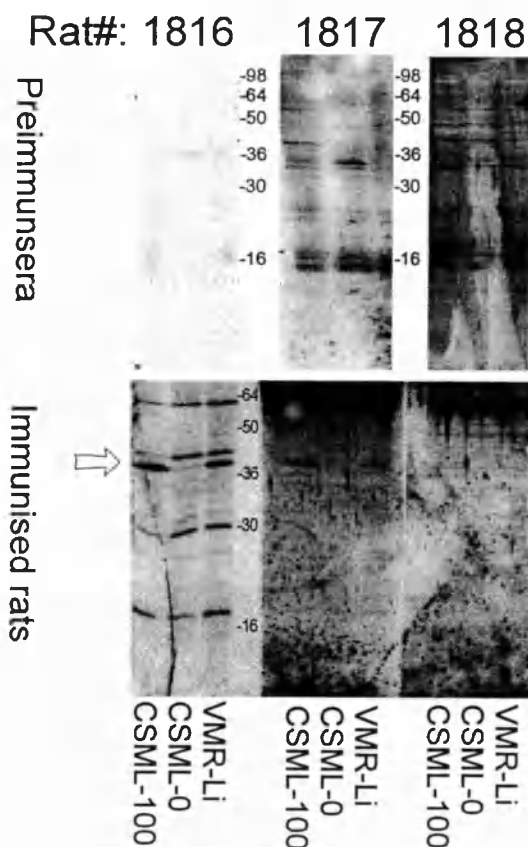
A



B



C



D

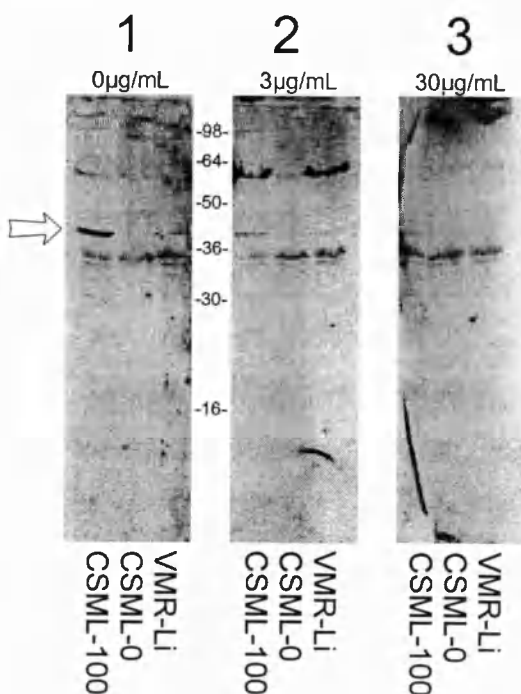


Figure 3.13: Production of polyclonal antibodies against E30 in rats. (A) The complete cDNA sequence containing the ATG-codon was inserted into the pGEX-30X-vector. The resulting fusion protein comprises the 26kDa *Schistosoma japonicum* glutathione-S-transferase (GST) part and E30. (B) Induction of fusion protein GST-E30 separated on a gel. Protein production induced with 0.1mM IPTG in the stated amount of time and thereafter harvested and purified (lane 4) and used for immunisation. (C) Western blot analysis of the activity of the immunised rat sera to E30 protein. The reactivity of the sera (diluted 1:100) of three rats was tested against app. 10.000 lysed cells of CSML-0, CSML-100 and VMR-Li prior to immunisation and following three immunisations and a boost. Serum from rat 1816 was selected for further experiments. (D) Blocking experiment performed to confirm the E30 reactivity. The activity to E30 was blocked using increasing amounts of purified fusion protein, from no fusion protein (blot #1) to 3 µg/mL (blot #2) to 30 µg/mL (blot #3). The suspected E30 protein is indicated with an arrow. Serum dilution was 1:200.

further E30 immune experiments. The background of this serum in Western blots was reduced when the dilution of the antibody was increased from 1:100 to 1:400.

To test the specificity of the obtained serum an inhibition experiment was performed (Fig. 3.13D). The reactivity against E30 seen in blot#1 in figure 3.13D was blocked with increasing amounts of purified fusion protein from lane 4 of figure 3.13B. Complete blocking of the specific binding to the protein of approximately 39kDa was observed after adding increasing amounts of recombinant fusion protein (3µg/mL in blot #2 to 30µg/mL in blot #3) as seen in blot#1-3.

E30 protein expression in tumour cell lines

To further elucidate the expression pattern on protein level the anti-E30 serum was tested against the panel of cells used in figure 3.8C using Western blot analysis (Fig. 3.14A). Protein expression was highest in the metastatic CSML-100, LL-Met, RAC34E and RAC311E, low in VMR-Ly and VMR-Li, and not detectable in the non-metastatic CSML-0 and the non-tumourigenic 10T½ and NIH3T3. Protein expression was not detected in RAC5E in contrast to the Northern blot analysis showing transcription. Otherwise the data from Western blot analysis confirmed the data from Northern blot analysis (Fig. 3.14A).

Evaluation of the expression pattern in various tissue could imply a possible function and substantiate a potential role in metastasis. To investigate the presence of the E30 protein in normal tissue of adult mice, Western blot experiments were performed with lysates from different organs. A protein of approximately 39kDa was detected in all samples tested (i.e. liver, lung, kidney, testis, brain, heart, thymus and spleen) except for salivary gland (Fig. 3.14B). Expression in brain was found in a separate Western blot analysis (data not shown).

Fractionation of CSML-100 cells was used to investigate the localisation of E30 (Fig. 3.14C). The cells were roughly fractionated into a cytoskeletal, a cytosolic and a nuclear fraction. Firstly, the cytosol was extracted with 0.1% Triton X-100, while the structure was retained with sucrose. Secondly, the cytoskeletal fraction was extracted with 0.1% NP-40 and calcium leaving the nuclear proteins in the pellet. E30 was detected in the

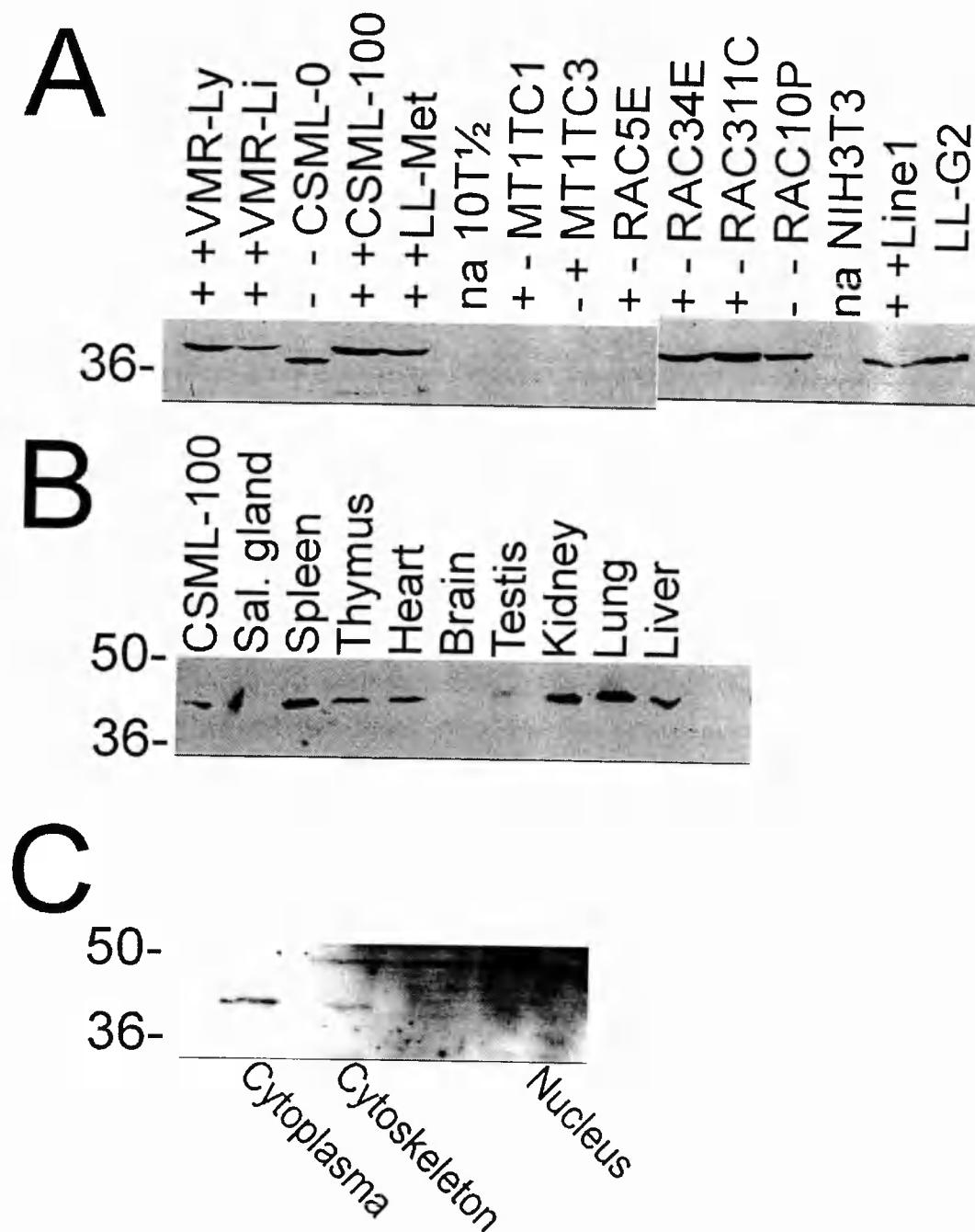


Figure 3.14: Western blotting comparing E30 protein expression. (A) Western blot analysis of cell lysates from a panel of mouse cell lines tested against anti-E30 serum. App. 10.000 cells/lane (adjusted individually using Coomassie-staining of gels) were separated on a gel, blotted to a filter and tested against anti E30-serum diluted 1:400. A 39kDa protein was stained in the majority of the metastatic cells. (B) Western blot analysis of tissue lysates. The presence of E30 was shown in spleen, thymus, heart, testis, kidney, lung and liver. The absence of signal in brain and the low amounts detected in testis was due to bad protein transfer in this area. A second Western blot experiment showed amounts equal to the other tissues of E30 protein in brain and testis (C) Fractionation of CSML-100 cells localised E30 protein to the cytoplasm. CSML-100 proteins were fractionated as described. E30 protein was found in the cytoplasmic and cytoskeletal fractions. In general the proteins were separated on a 12% SDS-PAGE gel, blotted and reacted with anti E30-serum (1:400 dilution).

cytosolic and cytoskeletal fractions, indicating that E30 was localised in the cytoplasm, possibly interacting with the cytoskeleton.

***In situ* hybridisation of E30 cDNA**

E30 transcripts detected by Western blot analysis showed that the E30 gene was expressed in various tissue. To show E30 transcript distribution in more detail, *in situ* hybridisation experiments were performed on sections of testis, uterus, spleen, thymus, brain, trachea, lung, oesophagus, stomach, skeletal muscle and decidua of day 8.5 and 16.5, including the placenta and all extraembryonic membranes.

***In situ* hybridisation of tissue**

The strongest signal was observed in spleen and thymus. Even short exposition times evolved strong signals in both organs (Fig. 3.15A). E30 transcript was expressed at more modest level in the testis. Figure 3.15D shows an accumulation of silver grains in the outer area of the seminiferous tubules. E30 signals were also detected in the epithelium and the lamina propria of the oesophagus and the stomach (Fig. 3.15H). In contrast the tunica muscularis was negative. Similar results were obtained from the trachea, where both the columnar epithelium and the lamina propria stained positive (data not shown). No signal was detected in the skeletal muscle. The hybridisations corresponded to Northern (data not shown) and Western blot results detecting E30 in testis, thymus, uterus, embryonic tissue and placenta. However, no *in situ* hybridisations could be detected in lung and liver, though these tissue show expression using Western blot analysis.

***In situ* hybridisation in embryos**

Data from ESTs and Northern blot experiments showed expression of E30 transcripts in embryo at several stages of development. To investigate in more detail the expression of E30 gene during mouse embryogenesis *in situ* hybridisation experiments on sagittal sections of day 12.5, 14.5 and 16.5 embryos were performed. At day 12.5 we detect the E30 transcript in the developing liver (Fig. 3.16A-C). At day 14.5 and 16.5

Figure 3.15: *In situ* hybridisation of the E30 gene in different tissues of the adult mouse. (A-C) Section of the thymus: medulla and cortex parts are shown. (A) Antisense hybridisation giving ubiquitous signal distribution. (B) Control hybridisation. (C) Hematoxylin-eosin(HE)-staining. (D-F) *In situ* hybridisation analysis of testicular tissue. (D) Antisense hybridisation detecting the E30 transcript in the outer layer of seminiferous tubules. (E) Sense hybridisation. (F) Antisense hybridisation of a seminiferous tubule at higher magnification. (G) The same region shown in F. (H-J) Detection of E30 transcripts in stomach. (H) Antisense hybridisation detecting E30 transcripts in the mucosal layer. (I) Sense hybridisation. (J) HE-staining. C, cortex; ge, germinal layer; lu, lumen; M, medulla; mu, mucosa; ow, outer wall; se, seminiferous tubules.

Figure 3.15

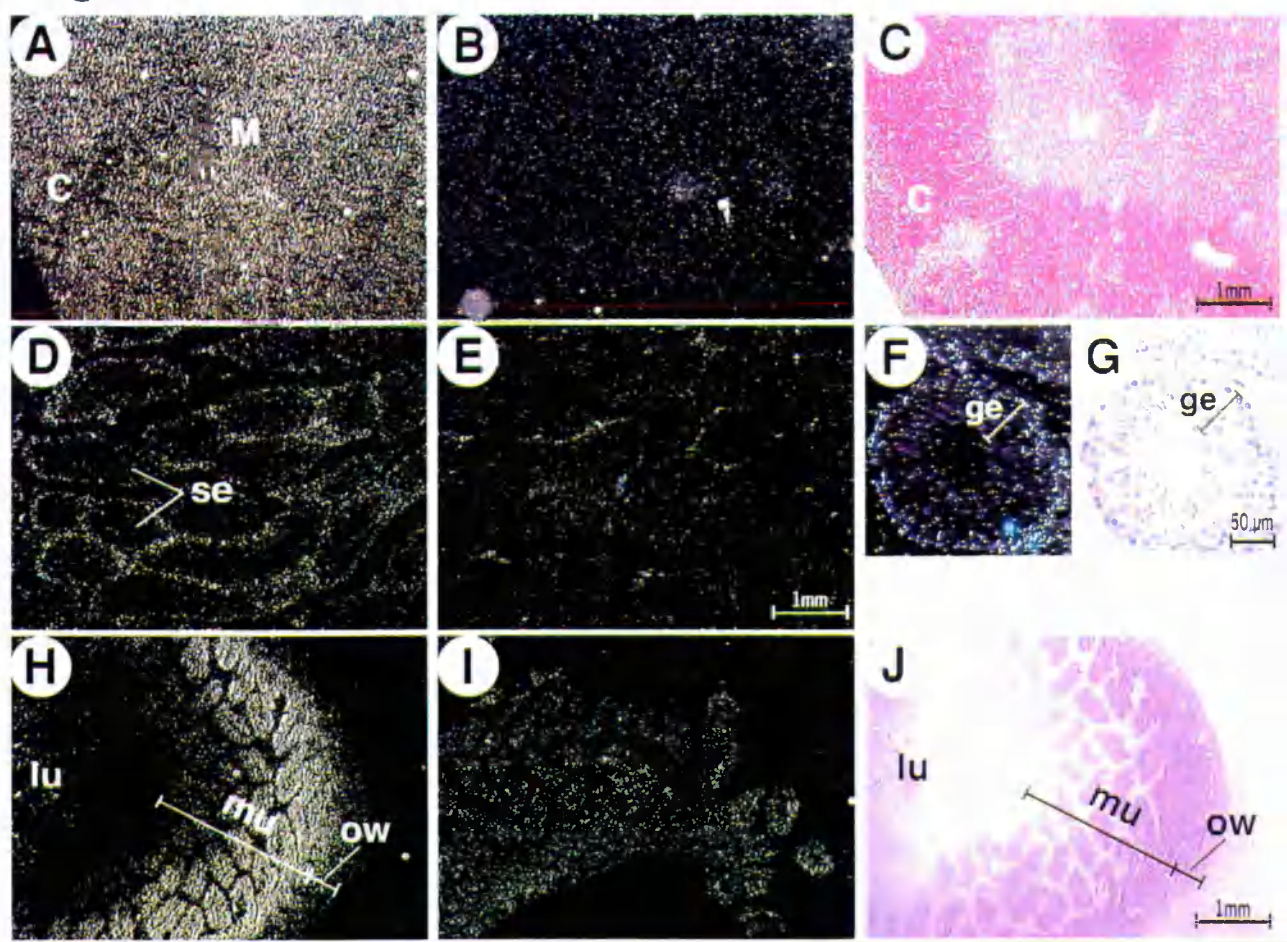


Figure 3.16

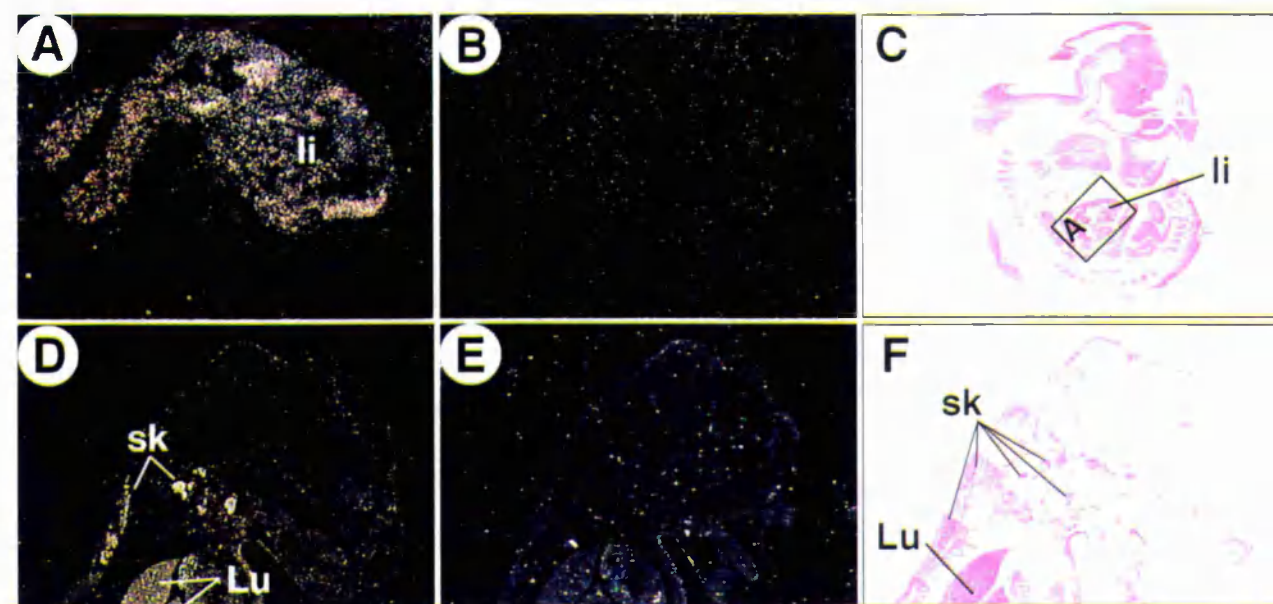


Figure 3.16: In situ hybridisation analysis of E30 transcript expression during mouse embryogenesis (A-C) Sagittal section through the trunk of a 12.5 dpc embryo (A) Strong signal show the liver. (B) Control hybridisation with E30 sense probe shows no signal. (C) HE-stained section; framed area is shown in (A). (D-F) Sagittal sections through a 16.5 day old embryo. (D) Showing upper part of the embryo with signals in the lung, skeletal muscle of the neck and muscle elements of the head. (E) Dark-field photomicrograph of an adjacent section to that in (D) hybridised with E30 sense probe show no signal. (F) HE-stained section of (D). ll, liver; Lu, lung; sk, skeletal muscle.

expression of E30 transcript was found in the developing lung and skeletal muscle of the neck and muscle elements of the head (Fig. 3.16D-F) but not in the liver.

***In situ* hybridisation of uterus and extraembryonic tissue**

Expression of E30 transcripts in uterus of a pregnant mouse (data not shown) encouraged *in situ* investigation of this organ. The preparation from the uterus of pregnant mice includes the deciduum with placenta and all extraembryonic membranes. *In situ* hybridisation of this tissue revealed a specific distribution of the E30 transcript. Figures 3.17A-C show sections of the uterus of a pregnant mouse (16.5 dpc). E30 transcripts were detectable in the epithelium lining the uterus lumen. In contrast, the uterus stroma showed no signal. Furthermore we could detect the E30 transcripts in the placenta, the yolk sac and the parietal endoderm (Fig. 3.17D-F). To obtain a more detailed pattern of expression, HE-counterstained sections were hybridised with the antisense probe. The accumulation of silver grains (black dots) is clearly visible on top of the inner layer (parietal endoderm) of the deciduum wall (Fig. 3.17H). Figure 3.17I shows E30 expression in yolk sac tissue. No signal was found in the amniotic tissue (note, that the tissue collapsed during preparation). The figure 3.17K shows a higher magnification of the yolk sac tissue, where it is forming a blood island. The yolk sac is composed of the inner embryonal mesenchyme originating from the mesoderm and the outer visceral endoderm of endodermal origin. The expression of E30 is restricted to the visceral endoderm, as the accumulation of grains indicated.

Figure 3.17: Expression of E30 in the uterus of a pregnant mouse and in extra embryonic tissue. (A-C) Transversal section through the uterus of mouse after 16.5 days of pregnancy. (A) Antisense hybridisation localises E30 transcripts in the uterus epithelium. (B) Control hybridisation. (C) HE staining. (D-E) section with extra embryonic tissue. (D) Antisense hybridisation showed signal in the placenta, yolk sac and parietal endoderm. (E) Sense hybridisation. (F) HE staining. (G-H) Antisense hybridisation of placenta and decidua wall. (H) Enlargement of a part of the decidua wall (boxed area in G). (I-J) Antisense and sense hybridisation showing signal in yolk sac, but not in amnion. (K) Enlargement showing grains in the visceral endoderm of the yolk sac. ep, epithelium; lu, lumen; pl, placenta; ys, yolk sac; pe, parietal endoderm; dw, decidua wall; am, amnion; em, embryonal mesenchyme; ve, visceral endoderm; bl, blood island.

Figure 3.17

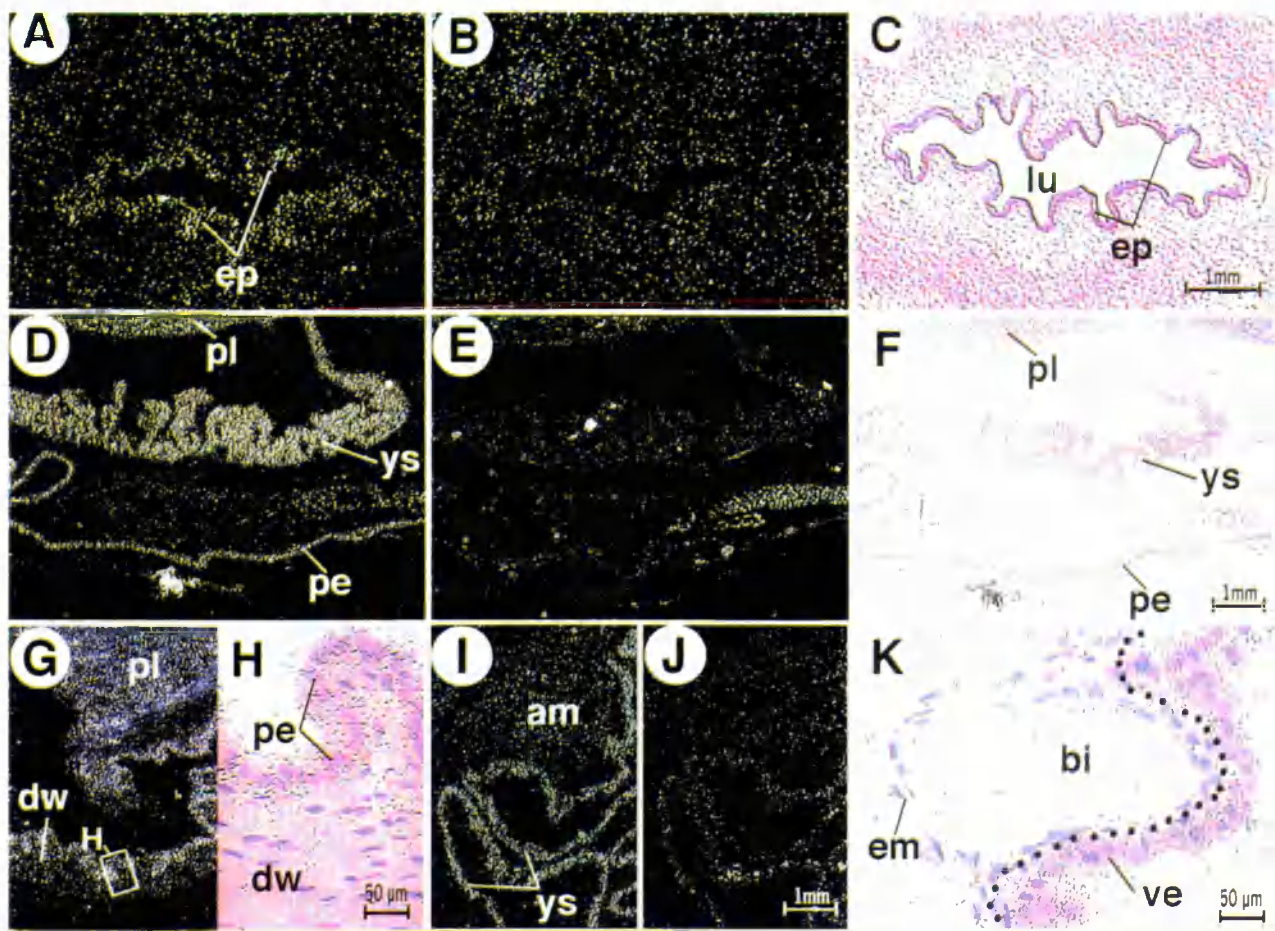


Figure 3.18 (part one)

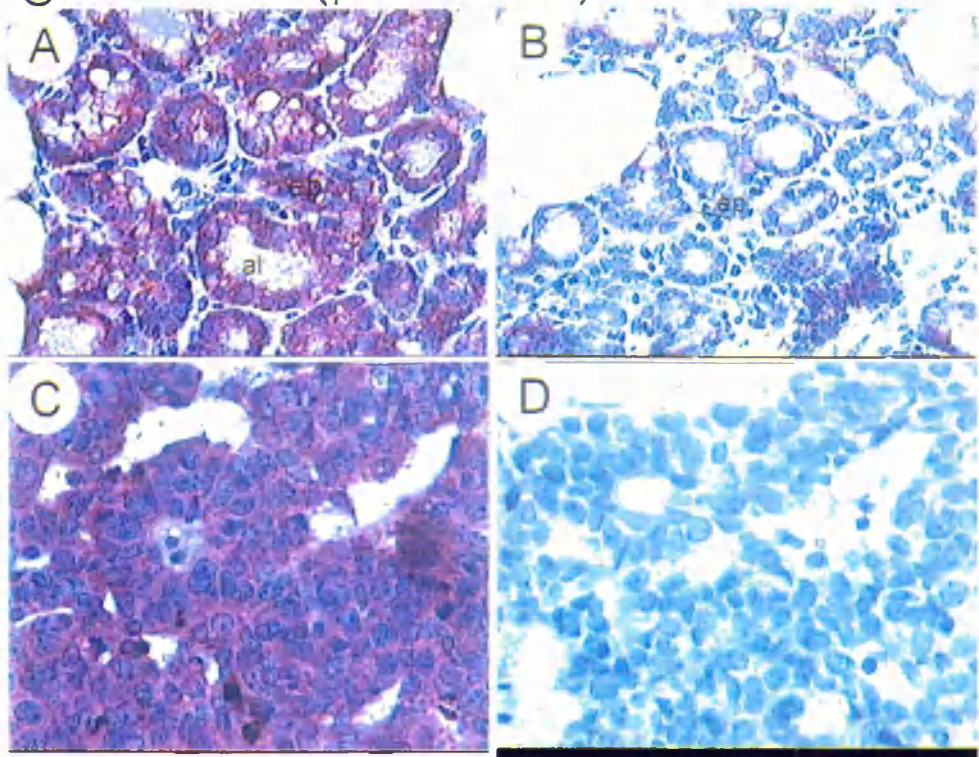


Figure 18 (continued)

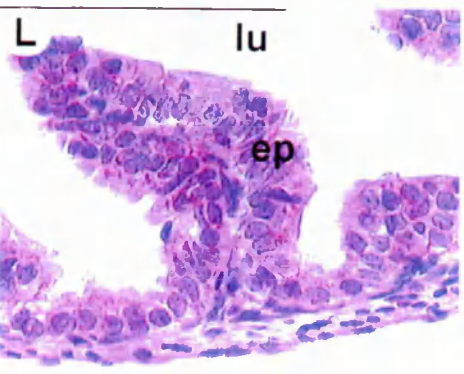
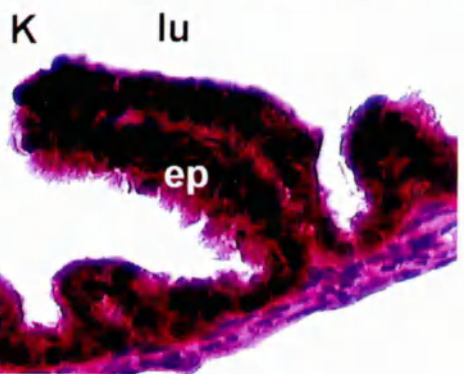
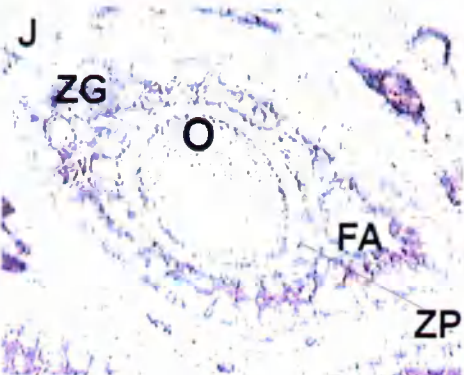
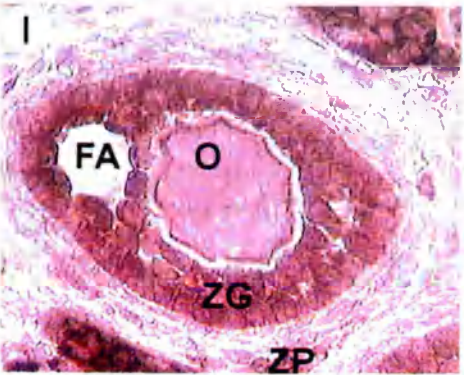
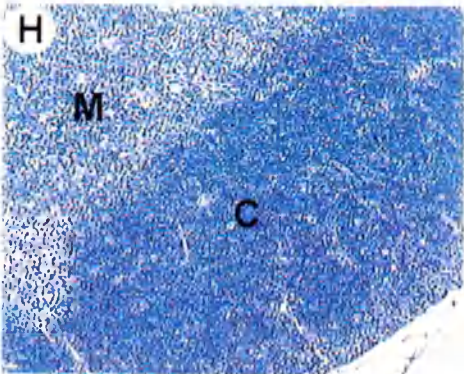
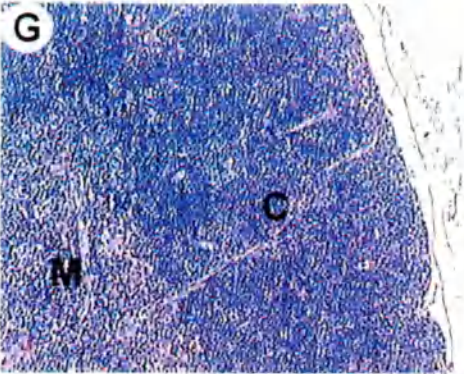
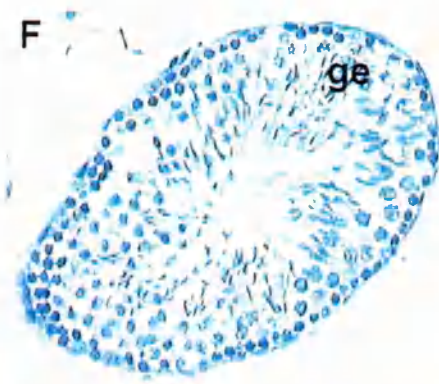


Figure 3.18: Immuno staining for E30 in mammary gland, enlarged mammary gland, testis, thymus, ovary and oviduct of the adult mouse. Immunostaining of the mammary gland. (A-B) . Immunostaining of enlarged mammary gland. (C-D) The epithelial cells in the enlarged area of mammary gland are stained. Immunostaining of testicular tissue. (E-F) Staining of the outer layer of seminiferous tubules. Magnification 200x. (G-H) Section of the thymus: medulla and cortex parts are shown. Magnification 100x (I-J) Secondary follicle. Staining of the zona granulosa and of the oocyte itself is seen. Magnification 400x. (K-L) Oviduct. The epithel lining of the oviduct, containing ciliated cells, is stained. Magnification 400x. A-H are hematoxylinstained, I-J are not counterstained, K-L are HE-stained. ge, germinal epithel, M, mucosa, C, cortex, O, oocyte, ZG, zona granulosa, FA, follicular antrum, ZP, zona pellucida.

Immunohistological staining with the anti-E30 antibody

The results obtained by immunohistochemical staining using the anti-E30 antibodies largely confirmed the data obtained by *in situ* hybridisation. The mammary, testis, liver, lung, ovary, oviduct, placenta, thymus and spleen were tested for expression of E30. There was staining in the epithelial layer of the mammary gland (Fig. 3.18A-B). Furthermore an enlarged mammary gland clearly stained positive (Fig. 3.18C-D). The E30 protein distribution in testis is coincident with the distribution of transcripts, where faint staining in the epithelium of the seminiferous tubules could be seen (Fig. 3.18E-F) in either spermatogonias, primary spermatocytes, myoids or Sertoli cells. In thymus staining was observed in cortex and medulla (Fig. 3.18G-H), however intensity was weak in contrast to the strong signal obtained by *in situ* hybridisation. A similar but even fainter signal was obtained after staining of the section of the spleen with the anti-E30 serum. Also the cells in and around the ovary stained i.e. the follicle cells in primary and secondary follicles and heavy staining of the oviduct could be observed. Also staining of the bile ducts of the liver was observed (data not shown).

Immunohistological staining of the sections prepared from the uterus and placenta including the extraembryonic tissue also confirmed the *in situ* hybridisation results. Expression of the E30 gene was seen in the epithelial lining of the lumen of the uterus (Fig. 3.19A-D), the visceral endoderm (Fig. 3.19E-G) of the yolk sac, the parietal endoderm of the deciduum wall (Fig. 3.19E, H-I) as well as in placenta (Fig. 3.19E).

Figure 3.19: Expression of E30 in the uterus of a pregnant mouse and in extra embryonic tissue. (A-D) Transversal section through the uterus of mouse after 16.5 days of pregnancy. (A) Immunostaining localises E30 to the uterus epithellum. Approximately 40x magnification. (B) Same as A without primary antibodies (C-D) Section of A and B, respectively magnified 400x. (E-I) Immunostaining of extraembryonic tissue. (E) Immunostaining showing staining of the placenta, yolk sac and parietal endoderm. Approximately 40x magnification. (F) Section showing staining in the visceral endoderm of the yolk sac. (G) Control staining without primary antibodies. Enlarged 400x. (H-I) 400x enlargement of the parietal endoderm (H) stained with anti-E30 serum and (I) stained with secondary antibodies alone. Abbreviations were as in Fig. 3.16. + and - indicate use of anti-E30 antibody or not.

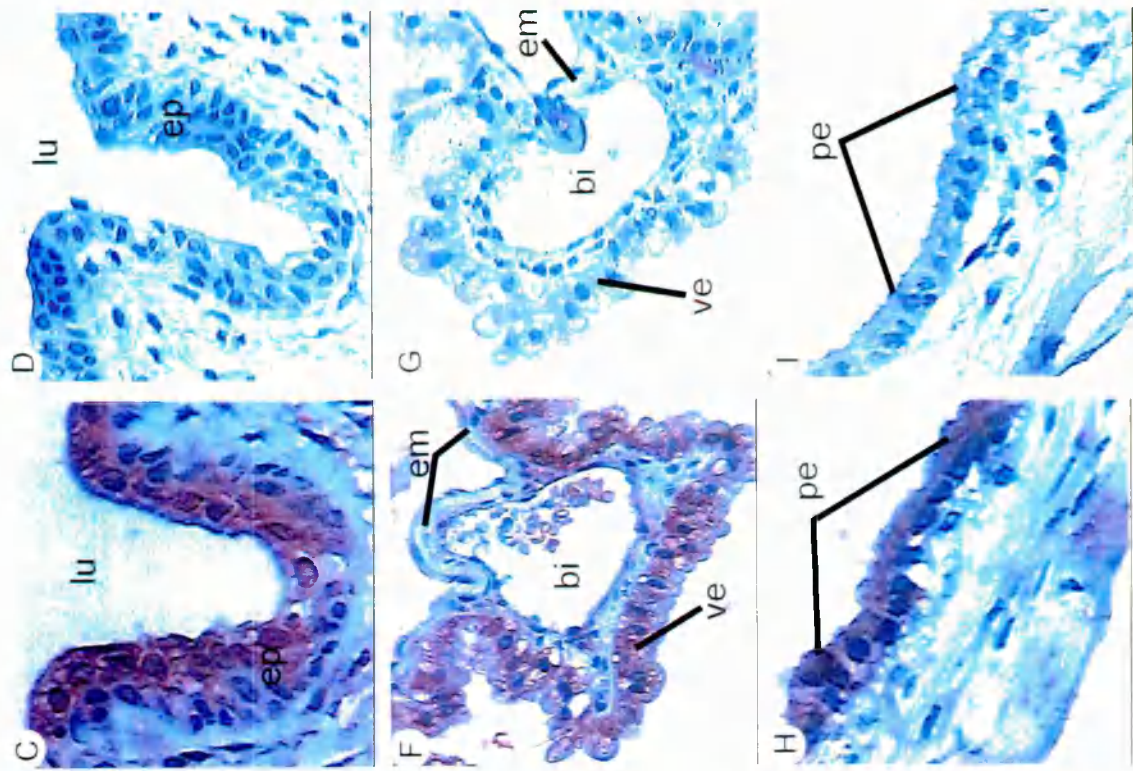
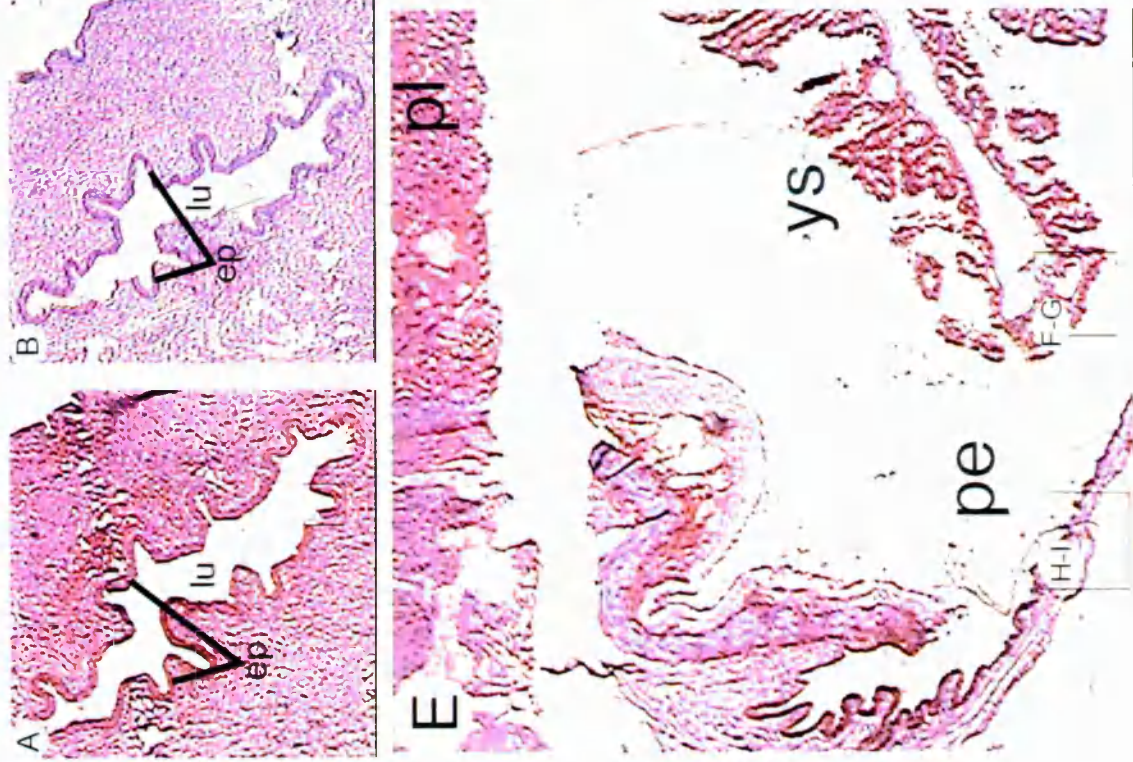


Figure 3.19

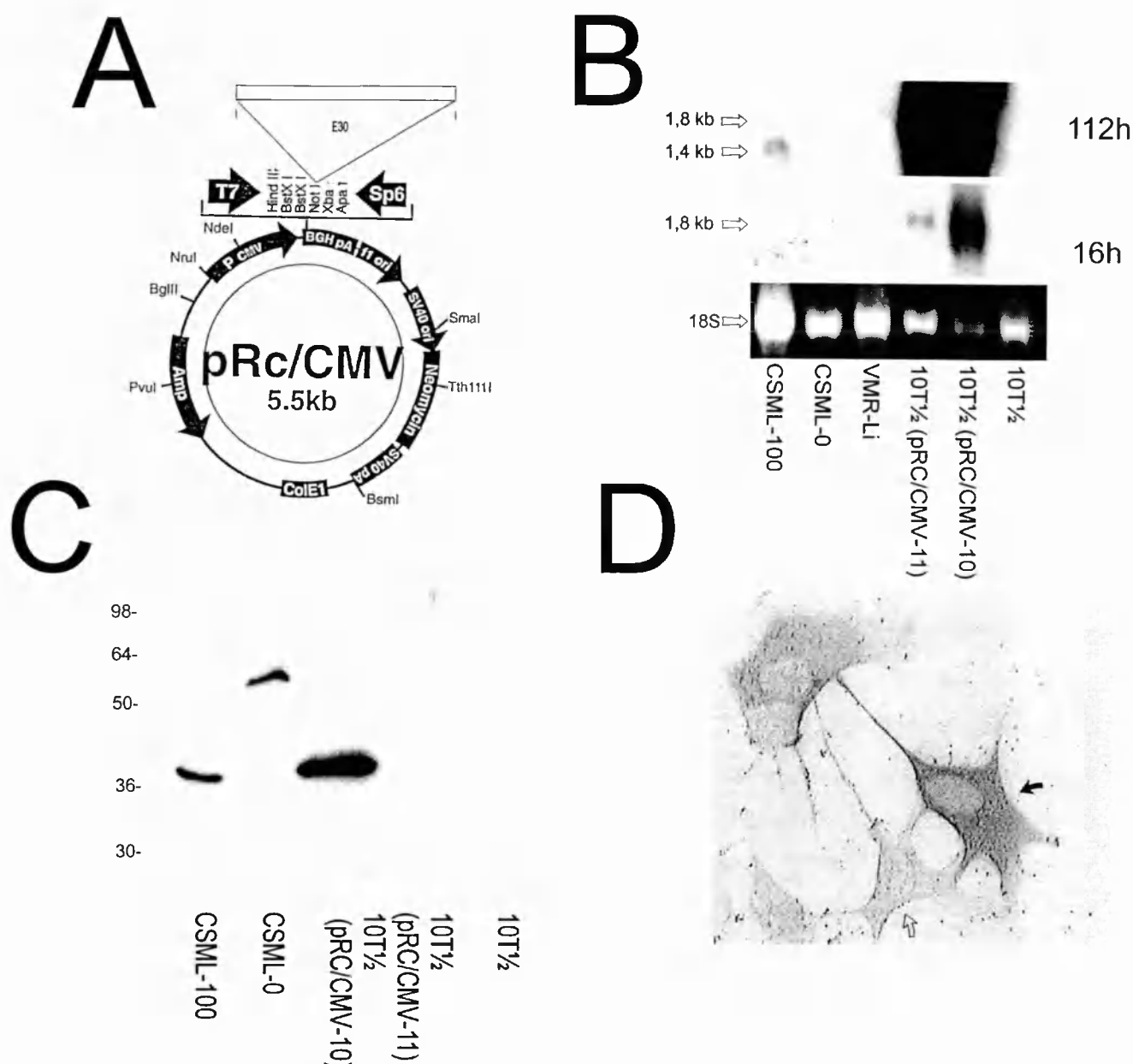


Figure 3.20: Transient transfection of 10T½-cells with pRc/CMV-10 lead to E30 expression.
 (A) The blunt-ended EcoRI cDNA sequence of E30 was cloned into pRc/CMV using NotI restricted, blunt-ended, dephosphorylated vector leading to a transcribed mRNA of the size of E30 initiating with the transcriptional initiation site provided by pRc/CMV and ending with the bovine growth hormone (BGH) polyadenylation signal. Cloning and sequencing lead to the isolation of a vector containing the E30 in a sense direction (pRc/CMV-10) and in an anti-sense direction (pRc/CMV-11). 10T½ cells were electrotransfected with the pRc/CMV-10 and pRc/CMV-11 vectors. After 24 hours the transfected cells were harvested (B and C) or stained (D). (B) Transcription of E30 was detected in transiently transfected 10T½ cells. A Northern blot was prepared with app. 10 µg total RNA per lane and transcription detected with labelled E30. The film was developed after over night exposure and after 3½ days revealing expression of mainly a 1.8 kb product in the transfected cells and a 1.4 kb product in CSML-100 cells. (C) Protein expression of E30 was detected in transiently transfected 10T½ cells. A Western blot using cell lysates of CSML-100, CSML-0 and 10T½ cells, non-transfected or transfected with sense and anti-sense expression vector, was performed. Equal amounts (app. 10.000 cells as judged from Ponceau staining of the filter after blotting) were separated on a 15% SDS-PAGE gel, blotted and stained with anti-E30 serum diluted 1:400. The size of the transfected E30 protein corresponds to CSML-100 expressed E30. (D) pRc/CMV-10 transfected 10T½ cells were stained with anti-E30 serum. After 24 hours cells were acetone fixed and stained with anti-E30 serum diluted 1:100. A stained cell (black arrow) next to an unstained cell (transparent arrow) is shown. App. 10% of the cells were stained.

Transfection of CSML-0 cells to express E30

Transient transfection

To test the suggestion that the E30 protein is involved in tumour progression, transient transfections of the non-tumourigenic 10T½ and the non-metastatic CSML-0 cell lines were performed (Fig. 3.20). 10T½ cells were transfected transiently to test the capability of the constructed vectors (pRc/CMV-10 and pRc/CMV-11) (Fig. 3.20A). These constructs consist of the pRc/CMV-vector and the EcoRI-fragment of the cDNA-clone including the presumed translation initiating ATG-site at nucleotide 23, the stop site at nucleotide 1036 and the degenerated polyA-signals all described under Sequence information. Northern blot analysis showed strong expression of E30 mRNA in sense transfected 10T½-cells, while the overexpression of antisense mRNA was less apparent (Fig. 3.20B). The size difference observed between the transfected E30 transcript of 1.8 kb and CSML-100 E30 mRNA of 1.4 kb could be due to utilisation of the transcriptional start in the vector situated 120 bases upstream of the insertion point and/or be due to usage of alternative signal for polyadenylation resulting in a prolonged 3' end. There was no difference in size of the E30 protein in transfected cells and the expressed protein in CSML-100 cells, indicating that E30 is translated in full length (Fig. 3.20C). E30 protein expression was detected in CSML-100 and pRc/CMV-10 transfected 10T½ cells, while neither normal 10T½ cells nor pRc/CMV-11 transfected 10T½ cells expressed E30. The sense transfected 10T½ cells overexpressing the E30 protein were stained by anti-E30 antibody shown in figure 3.20D. Approximately 10% of the sense transfected cells stained positive. Staining was restricted and concentrated to the cytoplasm. In contrast, the untransfected cells (Fig. 3.20D, unfilled arrow) and cells transfected with the antisense construct did not show staining (data not shown). There was no change in morphology between antisense and control transfected cells. Since 10% of the cells were transfected and the amount of protein expressed was about four times higher in sense transfected 10T½ cells than in CSML-100 cells, the average expression in transfected cells was increased approximately 40 times. An attempt to cyto stain CSML-100 cells with anti-E30 serum failed probably because of the limited affinity of the immune serum.

Stable transfection

Clones expressing the E30 protein after transfecting CSML-0 cells with the pRc/CMV-10 construct were obtained (Fig. 3.21). Western blot analysis of protein extract from

clones transfected pRc/CMV-10 was used to identify clones expressing E30 after 3-5 weeks in culture (Fig. 3.21A). However, retesting the expression of these cells approximately two weeks later showed loss of expression in eight cell lines. While nine clones were positive initially, three were positive two weeks later, with reduced expression in all cell lines. Subcloning of these cell lines led to the isolation of one clone, 3E7-9-C010, a stably transfected E30 expressing CSML-0 cell line (Fig. 3.21B and C). The level of E30 expression in this cell line was comparable to CSML-100 expression. As mentioned above, this level of expression was too low for cytochemical staining with the available polyclonal antibodies. The expression of E30 protein did not change the morphology of the cells. The metastatic ability of 3E7-9-C010 cells were tested in syngeneic A/sn mice.

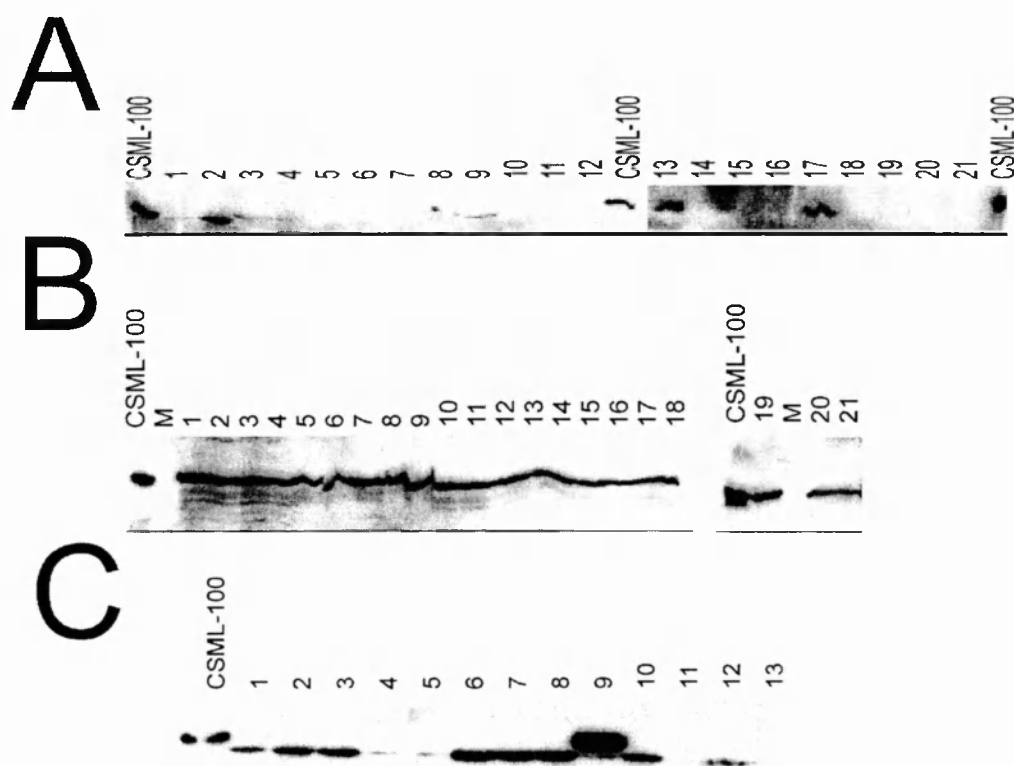


Figure 3.21: Stable transfections of CSML-0 cells. (A) Western blot of pRc/CMV-10 (lane 1-19) or pRc/CMV-11 (lane 20-21) transfected CSML-0 cells showing expression of E30 in lane 2, 8, 9, 13 and 17 (clone 1C6-C010, 2D7-C010, 2C11-C010, 3E7-C010, 3C3-C010) three weeks after transfection. E30 expression in CSML-100 cells was used as control. **(B)** Western blot of pRc/CMV-10 (lane 1-19) or pRc/CMV-11 (lane 20-21) transfected CSML-100 cells showing expression of E30 in all lanes. **(C)** Western blot of clones subcloned from E30 expressing 3E7-C010. Subclone number is indicated above the blot. Only subclone 9 (3E7-9-C010) expresses E30 protein. App. 10.000 cells were loaded pr. lane in all Western blots.

Spontaneous metastasis assay

Eight mice were injected sub cutis (sc) with 10^5 cells (Table 3.2). The mice were injected with either 3E7-9-C010 or CSML-0. The cells injected (>95% viable) gave rise to primary tumours in 2/3 3E7-9-C010 injected mice and 4/5 CSML-0 injected mice. The tendency of even small tumours from CSML-0 cells to become necrotic was unchanged for the transfected cells and there was no other obvious changes in the morphology of the primary tumours (Fig. 3.22A-D). Also the sizes of the tumours were unchanged with an approximately one-week delay of initiation of growth in mice injected with transfected cells (Table 3.2). Examination of lungs and liver did not reveal any microscopic metastatic lesions.

Table 3.2:

Spontaneous metastatic assay using transfected CSML-0 cells.

Mouse #	Cells injected	Experiment length ^{a)}	Initiation of tumour growth ^{a)}	Tumour size (mm)	Remark
2061	3E7-9-C010	-	-	0	No tumour
2062	3E7-9-C010	33	20	13	
2063	3E7-9-C010	20	20	8	
2076	CSML-0	-	-	0	No tumour
2077	CSML-0	20	20	11	
2078	CSML-0	20	20	12	
2079	CSML-0	20	13	14	
2080	CSML-0	14	13	14	

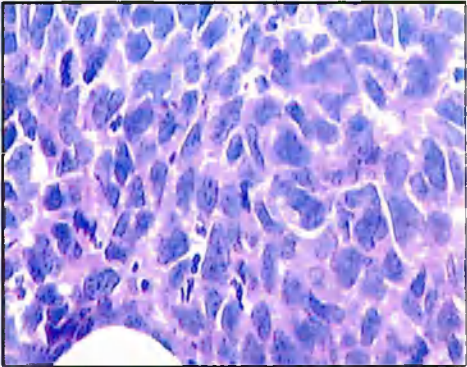
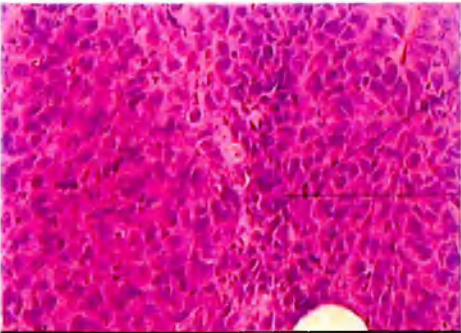
^{a)} Days after sc injection of cells.

Experimental metastasis assay

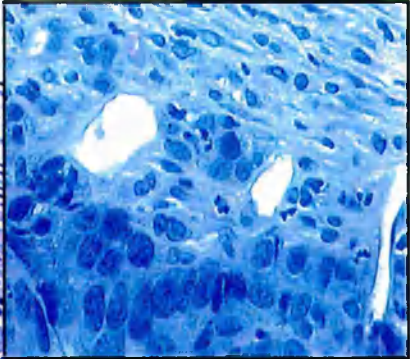
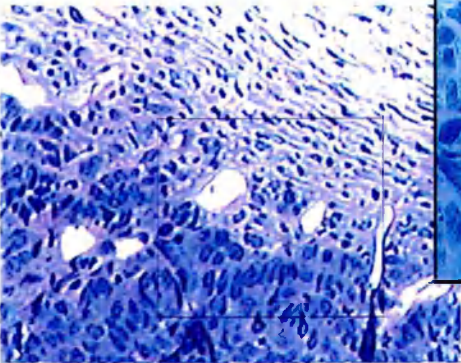
In the experimental metastasis assay three mice were injected intravenously (iv, into the tail vein) with 10^6 of 3E7-9-C010 or 8×10^5 of CSML-0 cells (Table 3.3). CSML-0 cells very rarely and not at all in this experiment produces metastasis in an experimental or spontaneous metastasis assay. When injecting 3E7-9-C010 cells, two mice collapsed with metastases in the lung and in the liver 64 and 69 days after injection, respectively. The first mouse was sacrificed after 33 days with no sign of metastatic growth. The volume of metastatic lesions was massive with several large nodules and a few small (Fig. 3.23 and 3.24). The volume of the tumour mass in the lung of the mice containing metastasis was comparable.

Figure 3.22: Hematoxylin-eosin stainings of primary tumours. Magnifications of approximately 100x and 400x of tumours arising from CSML-0 and 3E7-9-C010 are shown.

Figure 3.22



CSML-0



3E7-9-C010

Figure 3.23

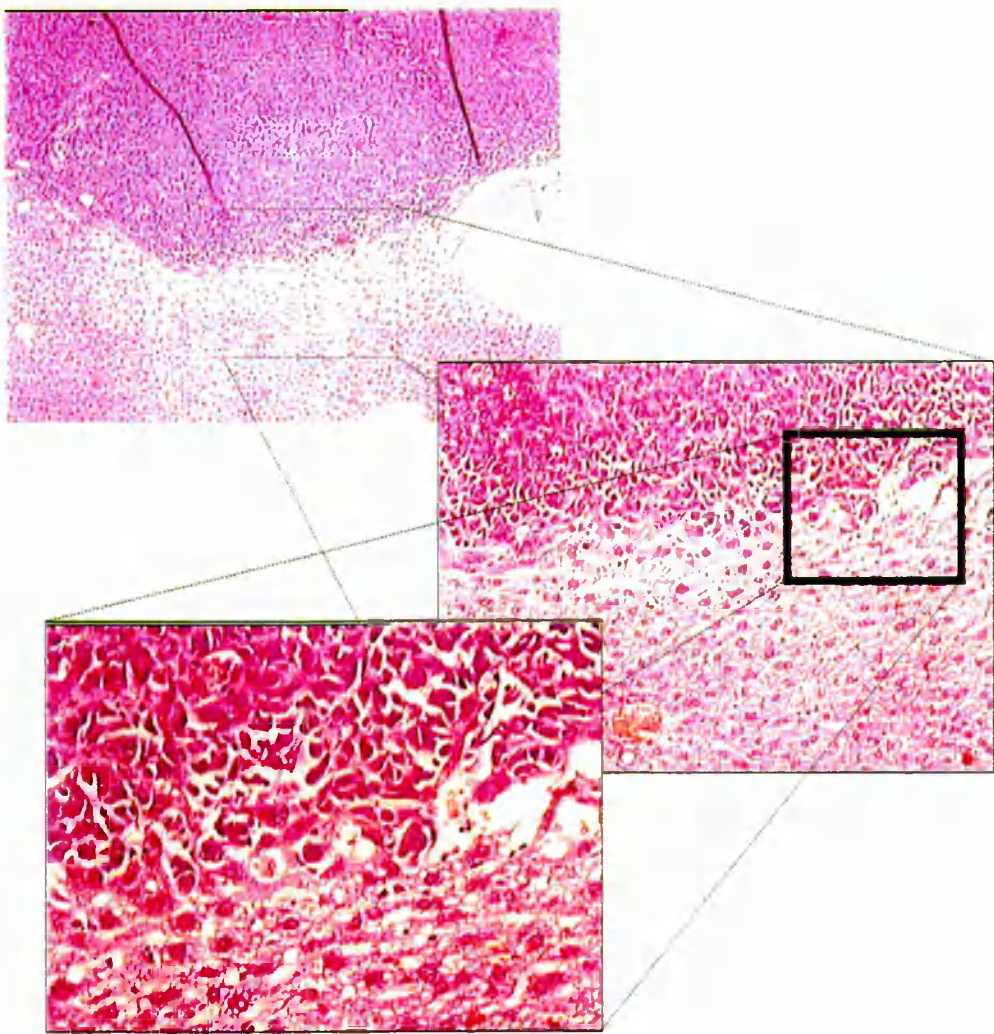


Figure 3.24

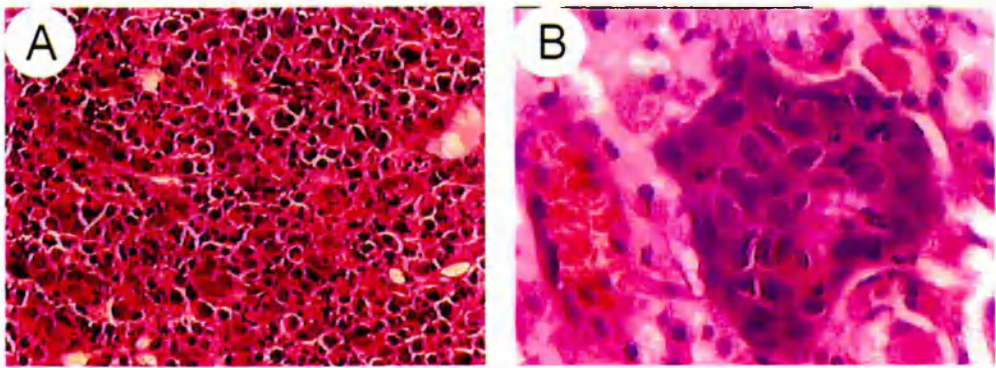


Figure 3.23: Hematoxylin-eosin stainings of metastasis in the liver of iv injected 3E7-9-C010 cells of mouse 2058. Sections of liver enhanced 100x, 200x or 400x. An invasive area of the metastatic lesion can be seen.

Figure 3.24: Metastatic lesions from 3E7-9-C010. (A) Center of a large lesion arisen in mouse 2059 enhanced 200x. (B) Small nodule in the lung from animal 2059 enhanced 400x

Table 3.3:

Experimental metastatic assay using transfected CSML-0 cells.

Mouse #	Cells injected	Days after injection	Number of metastasis
2058	3E7-9-C010	69	In lung>4 and liver=1
2059	3E7-9-C010	64	In lung>13 and liver=1
2060	3E7-9-C010	33	0
2051	CSML-0	85	0
2052	CSML-0	85	0
2053	CSML-0	85	0
2054	CSML-0	85	0
2055	CSML-0	85	0
2056	CSML-0	85	0

4. Discussion

In order to identify transcriptional differences we compared mRNA expression of two metastatic cell lines CSML-100 and VMR-Li to non-metastatic CSML-0. Using DD 56 fragments displaying differential expression were isolated. Ten of these fragments were retested, cloned and shown to be differentially expressed in Northern blot analysis. Six of them aligned to known genes (PACE4, Collagen XI α 1, MMTV, Lamin C, Ly-6A/E and Cystatin C), three fragments were homologous to partially sequenced murine cDNA (homologues to clones 9-1, 18-4 and 30-8 were found in libraries from mammary gland, brain tissue and miscellaneous, respectively), while one fragment lacked homology to published sequences (clone 32-9). The expression pattern of the fragments in 14 metastatic and non-metastatic cell lines pointed to a potential role of the corresponding genes in tumour progression. The possible role of the two genes expressed in CSML-0, i.e. PACE4 and Collagen XI α 1, in suppression of metastasis was shown for the first time. The role of Lamin C, Cystatin C and Ly-6A/E in metastasis has been suggested previously validating DD for the identification of differentially expressed genes involved in the metastatic process. Moreover, a new fragment (32-9), whose expression correlated with the metastatic potential of tumour cells, was described as well as the expression of three additional fragments shown to be EST-homologues. The coding region of one of these fragments was cloned and named E30. This clone contained an open reading frame with 338 aa, theoretically resulting in a 39kDa protein. Preliminary data indicated an effect of the expression of this gene in the metastatic process.

Differential display

The use of DD to identify differences between closely related cell lines with different metastatic potential was successful, although drawbacks were encountered. Consistent with our results, false positive clones have often been reported (155; 156), making reproductive runs and Northern blot confirmation necessary prior to selection of differentially expressed bands. Additionally, the suitability of the method depends on its ability to restrict the gene pool in a specific manner. The selection of 1/12 of the transcribed genes depends on the mRNA polyadenylation site being consistent i.e. of the sequence preceding the poly-A end to correspond to: one gene – one sequence. However, comparing sequences of the 3'-ends of known genes from the database with

the isolated cDNA, these sequences differed, indicating that the downstream primers selected 1/12 of the transcripts in the gene pool, but not 1/12 of the transcribed genes. The sequence of the cDNA clone of E30 confirmed this, since it when compared to clone 30-8 contained three extra 3' end nucleotides. As a consequence a few downstream primers and more upstream primers should be used to display a larger number of the transcribed genes.

It has been calculated, that a defined number of arbitrary upstream primer would display the entire population of a given cell line (157). In this set-up, however, displaying approximately 700 bands representing a maximum of 700 genes of the estimated 10-15,000 expressed cellular genes, led to the isolation of transcripts corresponding to MMTV and Lamin C gene, 5 and 4 times respectively. Expanding the DD, using additional primers, would increase this problem of isolating copies of identical genes.

Considering the above mentioned two points, displaying the entire pool of transcripts in the cell using DD might prove difficult. Compared to other methods like subtractive hybridisation and cDNA library screening, however, DD was a very efficient method for identifying differential gene transcription amongst closely related cell lines and it was possible to identify 17 reproducible differences, corresponding to 30% true positives, among the isolated 56 fragments. Extrapolation of the identified 10 differences, while displaying 700 gene fragments, leads to an estimated 200 differences between CSML-0 and CSML-100 cell lines assuming 14,000 expressed genes in the cells. Since seven out of 17 fragments isolated corresponding to 41% were copies of already identified differences, the 700 fragments represents less than 700 gene fragments, this 200 is an underestimate. A guess closer to the actual number of differences would be approximately double. This is in accordance with the recently applied SAGE technique, where 500 differences were found between normal prostate tissue and tumour tissue (158). None of the differences found in that study, match sequences described in the present study. However, the differences in species, progression state and tissue could explain the discrepancy.

Differential gene expression

Genes expressed in CSML-0

The differential expression of the transcripts in the CSML cell line system suggested a possible involvement in tumour progression for the ten different genes identified. We compared the expression of the identified gene fragments with genes of known metastatic potential. A correlation was found between the expression of the genes coding for PACE4 and Collagen XI α 1 as compared to E-cadherin expression, supporting an involvement of the identified genes in the suppression of tumour progression.

PACE4

PACE4 belongs to the KEX2/furin family of serine proteases. A function of the serine protease PACE4 in the suppression of tumour progression could be activation of proproteins in the cell. For example prohormones necessary for survival or proliferation, since PACE4 and other members of this family activate nerve growth factor (159), known to rescue serum-starved PC12 cells from apoptosis (160; 161). Also proMT1-MMP was cleaved *in vitro* by furin, head of the family of serine proteases to which PACE4 belong (162). It has been suggested that PACE4 is a tumour suppressor, due to a lack of expression in small cell lung cancer (163), and here we furthermore suggest an involvement in impeding tumour progression coinciding with this data.

Collagen XI α 1

The expression of Collagen XI α 1 has not previously been connected to cancer progression, either. Alternative splicing seen in CSML-0 cells occurs during development in tissue destined to become bone (164), in chondrocytes (150) and has been demonstrated in the human rhabdomyosarcoma cell lines RD and A204 (165). The binding of integrin β 1 to molecules of the ECM has been shown to salvage cells from programmed cell death (166; 167), and Collagen XI contains a binding site for integrin α -2 β -1 (168).

Genes expressed in CSML-100

We correlated the expression of genes coding for Lamin C, Cystatin C and ly-6A/E to the same panel of mouse cell lines. The expression is widespread with an increased expression in cell lines that are metastatic in both assays.

Lamin C

The ubiquitously expressed transcript of the nuclear envelope protein Lamin C was detected at lower levels in non-metastatic CSML-0, low metastatic MT1TC1 and RAC34E. The nuclear lamina in embryonic and lymphoid cells is composed exclusively of Lamin B (169) indicating that differential lamin expression may play a role in proliferation or differentiation. In testis cancer, upregulation of Lamin C in four out of four invasive non-seminoma tumours as compared with non-invasive carcinoma *in situ* points to a role in tumour malignancy (170), confirming a possible role of Lamin C in tumour progression.

Cystatin C

Transcripts of Cystatin C were widely expressed in the panel of mainly metastatic cell lines. Small amounts of transcript as well as protein could be detected in CSML-0 cells *in vitro*. Downregulation of Cystatin C protein expression seemed to occur in two RAC cell lines, RAC34E and RAC10P. The difference in expression in the CSML cell system was confirmed when tumours originating from CSML-100 and CSML-0 cells were stained. Cystatin C was mainly expressed in the tumour originating from cells of CSML-100. The non-focused nature of the staining could be due to the segregation of the protein. The exogenous Cystatin C is a potent inhibitor of a variety of cysteine proteases, the main target being the lysosomal cathepsin B. Cathepsin B has been implicated in metastasis through reports stating that the upregulation of the protease correlates with metastasis (171; 172), cathepsin B can degrade basement membrane *in vitro* (173), it participates in tissue remodelling *in vivo* (174), it can activate u-PA (88) and is expressed on the invasive edge of malignant tissue (92). We found overall expression of Cystatin C in the tumour comprised of metastasising CSML-100 cells and CSML-0 with higher expression in the CSML-100 tumour. The activity of Cathepsin B has been calibrated with the cysteine inhibitory activity in order to correlate the ratio of activity to malignancy (97; 172; 175). However, the cysteine inhibitory activity was in the majority of the studies

found to consist of Stefin A and B, two mainly intracellular cysteine protease inhibitors. It would be of interest to correlate the balance of expression of Cathepsin B and extracellular Cystatin C along with the localisation of the proteins in metastatic versus non-metastatic tumours.

Ly-6A/E

Ly-6A/E gene expression has been demonstrated in all but one cell line, which were metastatic in both assays (VMR-Ly, CSML-100, LL-Met, MT1TC3, but not VMR-Li), while it was undetectable in CSML-0, VMR-0 p9 and MT1TC1 (152). It is also found in the non-tumourigenic 10T $\frac{1}{2}$, the metastatic RAC5E and RAC34E and in low levels in the non-metastatic RAC10P. Ly-6A/E is a lymphocyte surface protein of unknown function. However, transformation experiments showing a correlation between Ly-6A/E protein expression and malignancy (176) and the high level of Ly-6A/E gene expression in metastatic cells, suggest involvement of the gene in tumour progression. A human Ly-6 homologue, CD59, has been shown to inhibit complementary destruction of the cell (177).

Unknown genes

We also identified three genes previously isolated as end sequenced tags (ESTs) and one gene not matching previously published sequences. Clone 9-1 appears to be of interest, since expression of this gene was increased in the metastatic VMR-Li, VMR-Ly and CSML-100, while reduced in corresponding low or non-metastatic partners. Clone 18-4 initially proved promising present in CSML-0 only, however, loss of expression in this cell line discouraged further investigation. Clone 32-9 was initially found in CSML-100 and VMR-Li, but not in CSML-0. Isolating cDNA-clones hybridised with this fragment resulted in the eight clones identical to galectin-1 (data not shown). These clones, however, did not contain any homology to clone 32-9. Clone 30-8 was expressed in the majority of the cell lines metastatic in the experimental and spontaneous assay indicating a role for the corresponding gene in metastasis:

E30:

Isolation of cDNA clone and characterisation

Clone 30-8 was used for screening a cDNA-library. A λ -phage cDNA clone of E30 (clone E30-8-2) was isolated. The sequence contained 1154 bp, while Northern blot analysis of E30 revealed a mRNA fragment approximately 1.4 kb in size. This discrepancy could be explained by lack of polyA-end in the cDNA-clone and missing nucleotides 5'. Estimating the size of the poly-A end to around 200 bp this allows for approximately 50 uncloned 5' nucleotides. Also the sequence of the potential translational initiation site conforms closely to the rules set out by Kozak *et al.* 1991 further supporting the cloning of the complete coding sequence. The polyadenylation signal was not the usual AATAA, but three sites mutated in one position situated less than 23 nucleotides from the 3' end (TATAA, GATAA, AATTA) might impose for a polyadenylation signal. Sequence analysis revealed an open reading frame spanning 1014 nucleotides resulting in a potential 338 aa protein with a predicted size of 39kDa. Comparison of native CSML-100 expressing endogenous E30 with CSML-0 cell line transfected with E30 expression vector, revealed no difference in gel mobility. Western blotting experiments showed that the polyclonal anti-E30 serum showed reactivity against a protein of the expected size (Fig. 3.13C). Transfection experiments confirmed the reactivity of anti-E30 serum displaying the occurrence of an approximately 39kDa protein. It was therefore concluded that cDNA clone E30-8-2 contained the complete coding sequence of the E30 gene.

The specificity of the polyclonal antibody was also addressed. Adding recombinant fusion protein to the assay blocked the reactivity against the supposed E30 protein. Also the expression pattern of E30 protein correlated with findings of mRNA expression. The transfection experiments demonstrated the appearance of a protein of the correct size in sense transfected cells. The results obtained by *in situ* hybridisations supported the immunohistological data. For example placenta, yolk sac, parietal endoderm, and the epithelial lining of the lumen in uterus were demonstrated to express E30 in both assays. However, the antibody did not stain native CSML-100 cells or tumours. This low sensitivity in immunocytochemical and immunohistological stainings impeded thorough investigation of the expression of E30 in tissue and transfected cells *in vitro* and *in vivo*.

In conclusion the antibody reacted specifically with E30 and was suitable for staining protein in Western blot analysis and not too demanding immunohistological stainings.

The E30 family of proteins

The protein sequence revealed an unknown protein. Extensive searches in various databases revealed no protein homologies. Also analysis of the sequence of the protein with respect to protein motifs, except for potential phosphorylation sites and N-glycosylation sites, failed to disclose functional data. However, EST fragments from *mus musculus*, *homo sapiens* and *caenorhabditis elegans* were found showing homology to the E30 sequence (Fig. 3.11 and Fig. 3.12). As mentioned previously, the information obtained from EST data was limited to a short sequence and the origin of the cDNA from which this short sequence was obtained. Hence, no functional data could be extracted from these data. Since all murine tags (23 ESTs in all) were 96% to 100% identical with E30, familywise E30 was the only expressed member found in mice. In *C. elegans* only a single EST-fragment homologous to E30 was described pointing to a protein, whose homologues could be preserved in various distantly related species. In human, four EST-fragments were found, overlapping each other, two by two. While the Jurkat and human intestine sequences C-terminally were 98% identical, the N-terminal human foetal heart and human liver were only 47% identical on DNA level and 59% identical on protein level. Though, EST-fragments are known to contain many sequencing errors, this difference indicates the existence of two human genes belonging to the E30 family of proteins.

Localisation of E30 in the cell

CSML-100 cells were fractionated into a cytoplasmic, cytosolic and nuclear fraction. By Western blot analysis E30 was detected mainly in the cytoplasmic fraction with small amounts detected in the cytoskeletal fraction. The cytoplasmic expression was also found in the cytostaining of the transient transfection. Whether a small amount was exported or transported to the nucleus would probably not be revealed using the 1816 anti-E30 serum in this assay. However, the cytoplasmic localisation was in concordance with protein sequence analysis, since neither a signal sequence nor a transmembrane region was discovered. E30 is therefore currently believed to be a cytoplasmic protein. The protein sequence of E30 did not reveal a possible function. The finding of *C. elegans* and human homologues indicated a function of E30 family proteins in distantly

related species. Five tryptophan residues N-terminal, at least one of them conserved, could indicate that this region of E30 is important for ligand binding.

E30 expression

Western blot analysis showed tissue expression in liver, thymus, spleen, lung, heart, brain, kidney and testis. Epithelial cells and lymphocytes were stained to test the expression in more detail. E30 was expressed in the following epithelial tissue: the epithelial lining of the uterus of pregnant mouse, the epithelial cells in the mammary gland and an enlargement therein, the mucosa of the stomach, the columnar epithelial lining of the oviduct, the epithelial part of the yolk sac, the epithelial layer of the deciduum wall, the columnar epithelium and the lamina propria of the trachea and the bile ducts of the liver. There are also indications for E30 expression in lymphocytes. First, E30 transcripts and proteins were found in spleen and thymus. Second, ESTs were isolated in lymph nodes and in T-cells.

Expression during embryogenesis indicates that E30 plays an important role in lung, liver and skeletal muscle development. Liver expression at 12.5dpc and expression in skeletal muscle and lung at 14.5dpc and 16.5dpc were detected as well as several ESTs from the blastocyst to 19.5dpc. The last general theme in the expression of E30 was expression in both the female and male reproductive system. In testis the outer layer of the germinal epithelia as well as the follicular cells of the primary and secondary follicle expressed E30.

E30 therefore was found to be especially abundant in highly proliferative epithelial cells in organs such as uterus and stomach and in embryonic and extraembryonic tissue. Also expression in mature lymphocytes (ESTs) and lymphatic tissue was observed.

Involvement in metastasis

Expression studies of E30 indicated an involvement in tumour progression in the CSML and Miller cells. Although E30 was found in libraries of the mammary gland, expression of this gene was downregulated in most non-metastatic adenocarcinoma derived cell lines by impeding transcription.

More importantly, the preliminary *in vivo* data of the transfected CSML-0 suggested an involvement of this gene in metastasis formation. While tumour formation was unchanged as compared to untransfected CSML-0 cells, sense transfected CSML-0 cells produced metastasis in the experimental assay. Metastasis was observed in two of the mice injected with 3E7-9-C010, with few but large metastases in the lung and liver. The reason for the lack of metastasis formation in the first mouse could be due to the short time allowed before it was sacrificed. Several experiments performed in the laboratory did not show a metastatic capability of CSML-0 cells. Hence, though no effect on formation of the primary tumour was observed, the ability to form metastasis was enhanced in sense transfected CSML-0 cells and the ability to spread to the liver was unique for these cells. Since cells when injected through the tail pass the lung before the liver, this indicated a potential of E30 to regulate metastasis formation in different organs.

General considerations when studying metastasis

The process of tumour cell spreading is complex. Its multi-step nature in the not fully understood mammalian organism, most probably offers many possible pathways when primary tumours spread. One of the *in vivo* assays most frequently used for testing metastasis is an end point assay used here detecting metastasis in various organs after injection of test cells either sc or iv. This assay is sensitive to changes leading to different results with the same cells in different laboratories (e.g. MT1TC1 and 4T07 cells were described as non-metastatic, but were found to be metastatic when tested in this laboratory). The rate-limiting step (intravasation, circulation, extravasation, proliferation) hindering metastasis of the non-metastatic cell is often unknown. It was shown that TIMP-1 transfected B-16 metastatic melanoma cells were rendered non-metastatic (80). However, intravital video microscopy demonstrated that the cells were capable of invasion, but unable to proliferate in the tissue. The cell line chosen to test a specific theory is of utmost importance, however, little is known about the rate limiting step of many of the non-metastatic cell lines and the metastatic pathways of the metastatic cell lines used in the laboratory today.

Since the number of differences between closely related cell lines with different metastatic potential could easily reach 500, it would be rather unlikely, via transfection of a single gene into the non-metastatic cell, to render it metastatic. If

possible this could either indicate: 1) extreme luck, 2) the existence of many available pathways for metastasis lacking just one protein, 3) the existence of one or more master genes switching on the metastatic process making the transfected gene one of these or an upstream target of this or 4) a balance of metastasis versus non-metastasis for the supposedly non-metastatic cell line, rendering it metastatic when given just a small extra advantage. If the last theory is confirmed transfection of the non-metastatic cell will have a good chance of success if the gene has metastatic potential and the non-metastatic cell is almost capable of metastasis. All in all, the selection of the right cell line, complemented for metastasis when transfected with E30, is crucial.

In this study we isolated fragments differentially expressed in the CSML cell line system and compared this expression with the MT1, RAC and VMR cell line systems. Several proteins, like E-cadherin, thought to induce or impede tumour progression have been identified (178). A 100% correlation of E-cadherin (as for the identified transcripts) can not be seen. Several additional experiments should be considered to further illuminate the potential role of the identified genes in tumour progression. Protein expression, localisation, proteases balanced by their inhibitors produced either in the stroma or by the tumour itself, should be addressed. The plasmin activator system consisting of several activators and inhibitors balancing each other in normal tissue (57) and the localisation of the protease or its inhibitor at the invasive edge or inside the tumour (58; 179) have been demonstrated for uPA, making its contribution to the metastatic process likely. To convincingly show the potential of the identified genes in the metastatic process, transfection experiments should be performed. Hence, thorough investigations are needed in order to include or exclude proteins as participants in the metastatic process.

5. Conclusion

In summary, the results demonstrate DD as an effective method for screening several cell types for differentially expressed mRNA. I identified genes not previously described in connection with metastasis and point to possible novel pathways of the metastatic process. Additional experiments should be performed to understand the contribution of these genes to the metastasis and the usefulness of the identified genes as diagnostic markers. Complete coding sequence of one of the identified genes,

named E30, was cloned. Transfection experiments of E30 demonstrated an involvement of this previously undescribed gene in metastasis.

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